

Sibel Yildirim

Dental Pulp Stem Cells



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Dental Pulp Stem Cells

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ISSN 2192-8118
ISBN 978-1-4614-5686-5
DOI 10.1007/978-1-4614-5687-2
Springer New York Heidelberg Dordrecht London

ISSN 2192-8126 (electronic)
ISBN 978-1-4614-5687-2 (eBook)

Library of Congress Control Number: 2012948961

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To Natasha McCarthy

Preface

The pace of scientific life continues to speed up considerably due to the flow of data being faster than ever. In my previous SpringerBrief's preface, I mentioned the groundbreaking discovery that neutrinos have been shown to exist at speeds faster than that of light. However, only a few months after this, the discovery team debunked their data by announcing that a loose fiber optic cable had introduced a delay in their timing system which explained the effect. Now a new subatomic particle consistent with the long-sought Higgs Boson has been discovered, despite the conservative approaches of many physicists.

Unfortunately, only those in the field commonly recognize key discoveries in cell biology. One of the unexpected discoveries in stem cell biology is induced pluripotent stem cell (iPSC), which shifted the long-held paradigms of biology to a new dimension. There are many more such discoveries in biology that have been published in high-impact journals without any discernible effect on society at all, even temporarily. For example, it has been shown that plant miRNAs could migrate into a host's blood and tissues via the normal intake of food. Eating "material" might mean eating the "information," which supports epigenetic regulation concepts. Moreover, stem cell fates are starting to be understood with the aid of nonlinear dynamics: mathematics is now working hand in hand with cell biology. These and many other discoveries have not attracted the importance they well deserve in wider society.

Media coverage referencing stem cell research and development is increasing rapidly to the extent that hardly a day goes by without hot-topic news items about them. This unfortunately also makes it very hard to distinguish fact from fiction. Creating bio-organs and almost magical possibilities to cure cancer and other critical diseases are featured regularly in the daily news. Likewise, for many dental practitioners, their wildest dream now is to build a new bio-tooth, aided by the magic of stem cells. Whether dental stem cells have any ability at all to generate a tooth now or in the future is not the main concern of this book. Rather, I am inviting the readers to understand that there are still many challenges waiting in the shadows of the dental stem cell field. For example, that dental pulp-stem cells are not mesenchymal, but mesectodermal stem cells.

The high costs incurred by dentistry education force its professionals to require high earning positions immediately after graduation. I believe this is the most important factor resulting in the rare occurrence of tooth-related discoveries, since dental surgeons with PhD qualifications are decreasing in numbers due to turbulent economic circumstances especially in developing countries. Also, compared to the late eighteenth and early nineteenth centuries, basic dental sciences are not so popular currently, worldwide.

In fact, teeth and the oral environment comprise one of the most sophisticated and complex adaptive systems of the human body. Teeth have to resist constant bacterial attacks from the mouth's environment, cope with extreme temperature changes, and withstand forces of considerably high pressure during chewing. Moreover, the rigid mineralized tissue of the tooth, dentin, surrounds the highly specialized mesenchymal tissue, dental pulp. The pulp–dentin complex creates an integrated organ that displays unique interactions between a mineralized and soft tissue with a significant regenerative capacity.

The very confined and small dentin–pulp organ provides enormous scope for the studies of development, differentiation, regeneration, immune-regulation/immune-modulation, epigenetics, genomics, proteomics, and other fields. Routinely discarded due to extraction, permanent and exfoliated primary teeth should be evaluated for these studies, since they are ethically and politically free human tissues. Additionally, they can be obtained noninvasively. Dental pulp cells easily provide access to suitable material for the study of cell biology's many aspects, including clinical applications.

I am deeply grateful to Bruce Rutherford for his generous and humble guidance during the whole of my scientific career. I am also grateful for the opportunity to have worked with Kamil Can Akçali, Jeremy Mao, Thimios Mitsiadis, Alp Can, Misako Nakashima, and with so many brilliant minds throughout my career. Thanks to John Dawson for his effort in reading the manuscript and sharing his brilliant ideas, even while on his once in a lifetime holiday. Thanks to my editor Aleta Kalkstein (Springer, USA) and assistant editor Renata Hutter for their supportive and kind assistance. I am very much appreciative of my family for their efforts, encouraging me to become a more down-to-earth person. And last but not least, thanks to Muammer Saglam for giving me an unquenchable flambeau and for his unconditional love that guided me to find my unique way.

Konya, Turkey

Sibel Yildirim

Contents

1 Dental Evolution.....	1
1.1 Historical Perspectives.....	1
2 Tooth Development	5
2.1 Stages of a Tooth Development	6
2.1.1 Initiation.....	7
2.1.2 Bud Stage.....	7
2.1.3 Cap Stage	8
2.1.4 Bell Stage.....	9
2.2 Odontoblast Differentiation	9
2.3 Dental Papilla and Follicle.....	12
2.4 Formation of Permanent Dentition	13
3 Dental Pulp Is a Connective Tissue	17
3.1 Histology of the Dental Pulp.....	17
3.2 Extracellular Matrix of Dental Pulp.....	17
3.3 Cells of the Dental Pulp	18
3.3.1 Odontoblasts	18
3.3.2 Fibroblasts.....	19
3.3.3 Undifferentiated Mesenchymal Cells.....	20
3.3.4 Immunocompetent Cells	20
3.4 Neuronal and Vascular Networks.....	21
3.5 Deciduous (Primary) Tooth Dental Pulp.....	21
4 Dental Pulp Stem Cells (DPSC)	25
4.1 Evidence for Dental Pulp Stem Cells: Emergence of a New Generation of Odontoblast-Like Cells	25
4.2 Origin of Progenitor Cells.....	26
4.3 Dental Pulp Stem Cells (DPSC)	27
4.4 DPSC from Deciduous Teeth Pulp	28
4.4.1 The Degree of Resorption of the Teeth Related to Stem Cell Isolation.....	29

4.5	Niche(s) in Dental Pulp.....	33
4.6	Epigenetics in Dental Pulp.....	36
5	Isolation Methods of Dental Pulp Stem Cells	41
5.1	Isolation of Dental Pulp Stem Cells.....	41
5.1.1	Enzyme Digestion Method	42
5.1.2	Outgrowth Method.....	42
5.2	Cell Culture Media for Dental Pulp Stem Cells.....	44
5.3	Cryopreservation of DPSC.....	49
6	Characterization of DPSC.....	53
6.1	Phenotypic Characteristics.....	53
6.2	Multidifferentiation Capacity.....	54
6.2.1	Problems in Adipogenesis.....	55
6.2.2	Odontogenic Differentiation	56
7	Reprogramming of DPSC to Induced Pluripotent Stem Cells	65
7.1	Is Reprogramming Necessary for Dental Regenerative Therapies?	68
8	Immunomodulatory Effects of DPSC	73
9	Dental Pulp Is a Complex Adaptive System	75
9.1	Changing Concepts in Cell Biology	75
9.2	The Tooth as a Complex Adaptive System	76
10	Conclusions	79
	About the Author	81
	Index.....	83

Chapter 1

Dental Evolution

Evolutionary and developmental biology are very complementary fields of research and study to that of comparative dental embryology, the latter arising in the last quarter of the nineteenth century. In order to achieve a suitable taxonomy for dental embryology, it is necessary to compare equivalent characteristics throughout existent and extinct species.

1.1 Historical Perspectives

Histological studies of dental organs started to emerge in the nineteenth century. According to Louise J. Baume in his seminal work titled “The Biology of Pulp and Dentin,” the demonstration of tubular structures in dentin first took place between 1835 and 1851 (Baume 1980). While the recognition of the pulp/dentin relationship followed those discoveries in 1852–1863, peritubular and nerve structures were not shown until later, between 1863 and 1868. The ontogenical aspects and phylogeny of dentin were taken to this stage of definition between 1867 and 1906 (Baume 1980).

From agnathans, jawless vertebrates, to mammals, observed modifications showed the evolution of mammalian dental ontogeny. Fossil teeth provided evidence to compare the relationships between extant and extinct groups and established phylogeny of mammals (Rougier and Novacek 1998). As highly mineralized appendages found in the entrance of the alimentary canal of both invertebrates and vertebrates, teeth have many functions such as processing of food, phonetic articulation, and defense in humans (Koussoulakou et al. 2009). The fact that dermal structures are the origin of teeth in vertebrates has many implications in dental biology. Those dermal structures have been thought to spread secondarily to the mouth, and there they became associated with bones (Butler 1995). Odontodes, which are superficial dermal structures located outside the mouth in jawless fish, were followed by buccal teeth that were localized to the jaw margins (Koussoulakou et al. 2009). Reif has brought attention to the similarity of teeth to the dermal denticles

(placoid scales) of elasmobranchs (Reif 1978), and Butler translates this knowledge by implying that teeth might have originated as products of the skin in jawless vertebrates, and furthermore that they subsequently spread to the mouth with the conversion of the mandibular arch into jaws in gnathostomes (vertebrate with jaws) (Butler 1995). Likewise, as the ancestor of teeth, odontodes was similar to placoid scales of recent sharks.

Most nonmammalian vertebrates have *homodont* dentition (all teeth of the same type). Human dentition is *heterodont* and *diphyodont*, which includes deciduous and permanent succession. While the dentition of animals with only one set of teeth throughout life is *monophyodont*, continuously replacing dentitions are *polyphyodont* (Fraser et al. 2006). The reduction in teeth number and generations (from polyphyodonty to oligodonty) and the increase in morphological complexity of teeth (from homodonty to heterodonty) represent important factors for mammalian diversification (Koussoulakou et al. 2009; Salazar-Ciudad and Jernvall 2010).

The reappearance of some teeth, which were lost over time, in birds is an interesting evolutionary concept (Chen et al. 2000). One of the central evolutionary principles known as Dollo's law indicates that an organism is unable to return, even partially, to a previous stage already realized in the ranks of its ancestors (Tomic and Meyer-Rochow 2011). Obviously, under appropriate conditions, the lost odontogenic capacity of avian ectomesenchyme can be regained (Louchart and Viriot 2011). Possible explanations for this are the preserved odontogenic capacity in oral epithelium or a safeguard mechanism such as gene redundancy, since knockout mutations of most teeth-related genes do not cause developmental arrest. Moreover, those mutations cannot prevent the formation of dental lamina, which can be observed in birds, although their teeth were lost over 60 millions years ago (Chen et al. 2000; Koussoulakou et al. 2009). Understanding the rules governing the reacquisition of lost properties will provide a wide range of clinical and biological applications to impaired or lost teeth (Koussoulakou et al. 2009).

Mammalian dentition consists of teeth that form as separate units, and the linear arrangement of teeth at the jaw margins shows spatial repetition. Butler hypothesized that a morphogenetic field determined forms of the teeth (Butler 1967). This concept was adapted to human dentition by Dahlberg (1965) who proposed that there was a field of influence operating on each of the tooth classes, i.e., incisors, canines, premolars, and molars. Later, the clone theory of dental development was proposed by Osborn (1974). He postulated that an inhibitory zone exists around the developing tooth germ, and that the germ can act as an attractor. This attractor acts like a pool, which attracts molecules from surrounding tissue or tooth germ itself (Osborn 1974). Additionally, according to the reaction/diffusion model, interactions between activator and inhibitor molecules such as FGF8/BMP4 and ectodin/BMP4 can give repeated structures (Neubuser et al. 1997; Laurikkala et al. 2003). Moreover, the multi-attractor concept can easily be applied to the developing tooth germ (Zhou et al. 2011). Sharpe's ideas about odontogenic homeobox code are trying to explain how different tooth classes are initiated in different parts of the oral cavity in response to molecular cues and the expression of specific groups of homeobox genes (Sharpe 1995). Recently, the complementary actions of the field, clone, and

homeobox code models have been proposed and a single incorporated model for dental patterning was hypothesized (Mitsiadis and Smith 2006). Such an unifying view can be extended into the clinical settings using findings from dental patterning in individuals with missing and extra teeth (Townsend et al. 2009).

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Chapter 2

Tooth Development

Experimental research on tooth development or odontogenesis is based very largely on the teeth of murine rodents (Butler 1967). Pioneering work by Shirley Glasstone on rat tooth germ cultures gave detailed information about structural, histological, and self-differentiating interactions of explants (Glasstone 1936). However, the dentition of the mouse and that of many other mammals is very different. Mice have highly specialized incisors, distinctive molar patterns, a small number of teeth, and they do not have tooth replacement (Butler 1967).

In vertebrate embryos, a group of cells, called neural crest (NC) cells, separate from the neural tube and migrate away from their parental epithelium to reaggregate with other cells. In the developing embryo, almost all organs, glands, and tissues, such as craniofacial skeleton, cornea, teeth/dentin, thyroid gland, thymus, cardiac septa, adrenal gland, melanocyte, autonomic nerve, sensory nerve, and Schwann cells, have these basic cells (Crane and Trainor 2006; Alberts et al. 2008; Lee et al. 2010a). In addition to the unique invasiveness of NC cells, their contribution toward building the head of vertebrates has been considered to be a turning point in the evolution of the vertebrates (Gans and Northcutt 1983).

Although NC cells are of ectodermal origin, it has been suggested to call them “mesectoderm” or “ectomesenchyme” since they undergo “mesenchymalization.” This property is important to discussions regarding mesenchymal stem cells since their origin is mesenchyme, which is derived from the mesodermal germ layer (Le Douarin et al. 2004). On the other hand, along with the cranial skeleton and other tissues of the head and neck, odontoblasts and tooth papillae are derived from mesectoderm or ectomesenchym (Le Douarin et al. 2004). Oral ectomesenchymal and ectodermal inductive interactions are the earliest expressions recorded in vertebrate fossils. These epithelial (oral ectoderm) and mesenchymal (ectomesenchyme/mesectoderm) interactions phylogenetically precede the origin of odontogenesis (Moss 1969).

Teeth share similarities with the other ectodermal organs, such as hair, feathers, scales, beaks, and many exocrine organs, in the placode and bud stage. These similarities disappear by morphogenesis. Initiation of tooth development includes a series of sequential reciprocal inductive molecular interactions between the dental epithelium and the underlying ectomesenchymal

cells (Jernvall et al. 2000). While the first signal inducing differentiation comes from the mesenchyme in all ectodermal organs, in tooth development, morphogenetic events are started by the signals from the epithelium (Pispa and Thesleff 2003). Several recombination studies revealed that only the very early first arch epithelium cells (occurring during the 8–11.5th embryonic days (ED) of mouse development) and ectomesenchyme (ED 12) have odontogenic potential (Mina and Kollar 1987; Lemus 1995).

The molecular aspects of tooth development are similar to those in the development of other organs and include epithelial–mesenchymal interactions. A plethora of molecules (approximately 300) are involved in tooth development such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), sonic hedgehog (SHH), and wingless integrated (WNTs). As mentioned previously, BMP4 acts antagonistically with FGF8 and this interaction plays a role in the periodic patterning (Neubuser et al. 1997). Irma Thesleff's group from the University of Helsinki has been working on the key features of dental development. Their web site, <http://bite-it.helsinki.fi/>, includes a wide range of data largely originating in previously published reports and is thus a compilation of the work of the researchers in this field. The web site provide a source for the expressions of growth factors, receptors, signaling molecules, transcription factors, intracellular molecules, extracellular molecules, and plasma membrane molecules during the different stages of tooth development in mice, rats, humans, and other species. The molecular dialogue between oral ectoderm and odontogenic mesenchyme during tooth development is very complicated (Fig. 2.1) (Jernvall and Thesleff 2000). There is a vast knowledge about the signal interchanges that are crucial to control differentiation and morphological changes and spatiotemporal expression of specific genes during teeth formation, yet little is known about the regulation of the signals (i.e., down or up) (Tucker and Sharpe 2004). All of the data show that there is no single gene that is directly connected with ontogenesis or the lack of any specific tooth. Instead, tooth initiation and morphogenesis occur by an orchestration of numerous genetic and epigenetic factors (Jernvall and Thesleff 2000; Koussoulakou et al. 2009). At the same time, most of the developmental defects in teeth usually occur as a result of mutations in genes encoding signaling molecules and transcription factors (Koussoulakou et al. 2009), such as mutations in the *PAX9* gene resulting in partial or total anadontia and mutations in *RUNX2* causing supernumerary teeth (Peters et al. 1998; Ryoo et al. 2010).

2.1 Stages of a Tooth Development

Each tooth passes through four morphological stages: initiation, bud, cap, and cell stages (Fig. 2.2) (Tucker and Sharpe 2004).

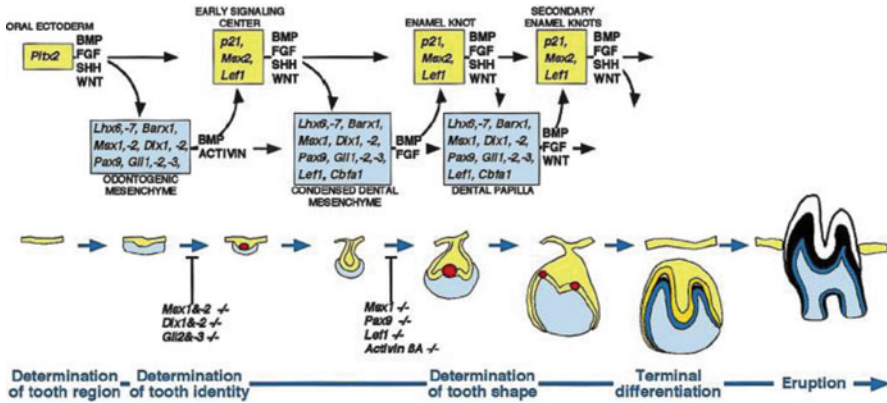


Fig. 2.1 Schematic representation of the signal and transcription factors mediating the reciprocal signaling between epithelium and mesenchyme during advancing tooth development. The molecular cascades are shown *above* and the corresponding morphological stages *below*. The transcription factors and signals considered to be important for particular developmental stages are indicated in the squares and above the arrows, respectively. Note how the same signaling pathways are used reiteratively during advancing tooth development, and how tooth development arrests in the knock-out mouse experiments to the early signaling center or the enamel knot stage. Key: yellow, tooth epithelium; red, enamel knots; blue, tooth mesenchyme (reproduced from Jernvall and Thesleff 2000 with the permission of the publisher)

2.1.1 Initiation

The initiation of tooth begins at the end of the fifth week of human gestation and ED 10 of mouse development. A localized thickening or placodes within the primary epithelial bands, formed after about 37 days of development, initiate tooth development. In a subdivision of the primary epithelial band, the dental lamina, localized proliferative activity leads epithelial outgrowths into the ectomesenchyme. Since the underlying ectomesenchyme is more active than the epithelial cells, these ectomesenchymal cells accumulate the epithelial outgrowths soon afterwards. As those cells fold, the forming structure proceeds as per the following descriptive morphological stages of tooth development: bud, cap, and bell. Folding and growth of the epithelium give the final shape of the tooth crown (Fig. 2.2) (Tucker and Sharpe 2004; Nanci 2008).

2.1.2 Bud Stage

This stage occurs between the 7th and 9th weeks of human gestation and ED 11–13.5 in mice embryos. It is represented by the first epithelial invagination into the oral ectomesenchyme. The internal part of the tooth bud contains star-like shaped, glycosaminoglycan synthesizing stellate reticulum cells. Some cells within the stellate reticulum in mice have been identified as putative stem cells (Bluteau et al. 2008). Odontogenic

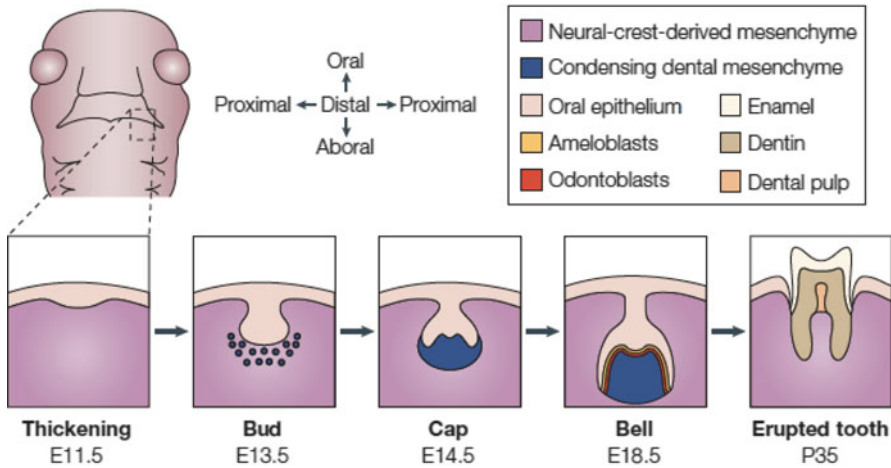


Fig. 2.2 Stages of tooth development. A schematic frontal view of an embryo head at ED 11.5 is shown with a *dashed box* to indicate the site where the lower (mandibular) molars will form. Below, the stages of tooth development are laid out from the first signs of thickening at ED 11.5 to eruption of the tooth at around 5 weeks after birth. The tooth germ is formed from the oral epithelium and NC-derived mesenchyme. At the bell stage of development, the ameloblasts and odontoblasts form in adjacent layers at the site of interaction between the epithelium and mesenchyme. These layers produce the enamel and dentin of the fully formed tooth (reproduced from Tucker and Sharpe 2004 with the permission of the publisher)

potential is switched from the epithelium to ectomesenchyme during the bud stage. While many ectodermal organs such as exocrine glands, hair follicles, beaks, and teeth share morphological similarity in the bud stage, different ectodermal organs become specific from the beginning of bud-to-cap transition (Jernvall et al. 2000).

2.1.3 Cap Stage

The tooth bud transforms into a cap by differential proliferation and in-folding of the epithelium (Koussoulakou et al. 2009). As the epithelial bud cells proliferate, ectomesenchymal cells condense and morphological differences between tooth germs begin during the cap stage. At ED 12, the histologically distinct epithelial mass, called the enamel knot, is induced by WNT and BMP4. Enamel knot cells do not show cell division and after their transient organizing role is complete, they go apoptosis at the end of the bell stage (ED 16) (Jernvall et al. 1998). Histo-differentiation begins late in the cap stage and in the next bell stage the cells of the crown *ameloblasts* and *odontoblasts* are differentiated. A single layer of columnar cells, which border the dental papilla and reside inside the cap, is called *inner dental epithelium* (IDE). The outer part of the cap is covered by the *outer dental epithelium* (ODE) (Marson et al. 2008). While the cap-shaped epithelial growth is widely referred to as *enamel organ*, the condensed ectomesenchymal cells are referred as *dental papilla*. The *dental follicle*

covers the outside of these two substances. The enamel organ, dental papilla, and dental follicle constitute the tooth germ. The dental papilla is separated from the enamel organ by a basal lamina and is located between IDE and undifferentiated mesenchymal cells of the papilla.

2.1.4 Bell Stage

Terminal differentiation of ameloblasts from IDE and odontoblasts from mesenchymal cells of dental papilla, and the formation of two principal hard tissues of the tooth, enamel, and dentin, are initiated during the bell stage. Ameloblast and odontoblast differentiation is regulated by interactions between the epithelium and mesenchyme (D'Souza 2002; Nanci 2008). While dental papilla is the origin of the future dental pulp, dental follicles give rise to cementoblasts, osteoblasts, and fibroblasts. In conclusion, NC cells give rise to dentin-producing cells, odontoblasts; cementoblasts, which produce root dentin covering; osteoblasts, which participate in the formation of dental alveoli; and fibroblasts, which synthesize collagen for periodontal ligaments (Fig. 2.3).

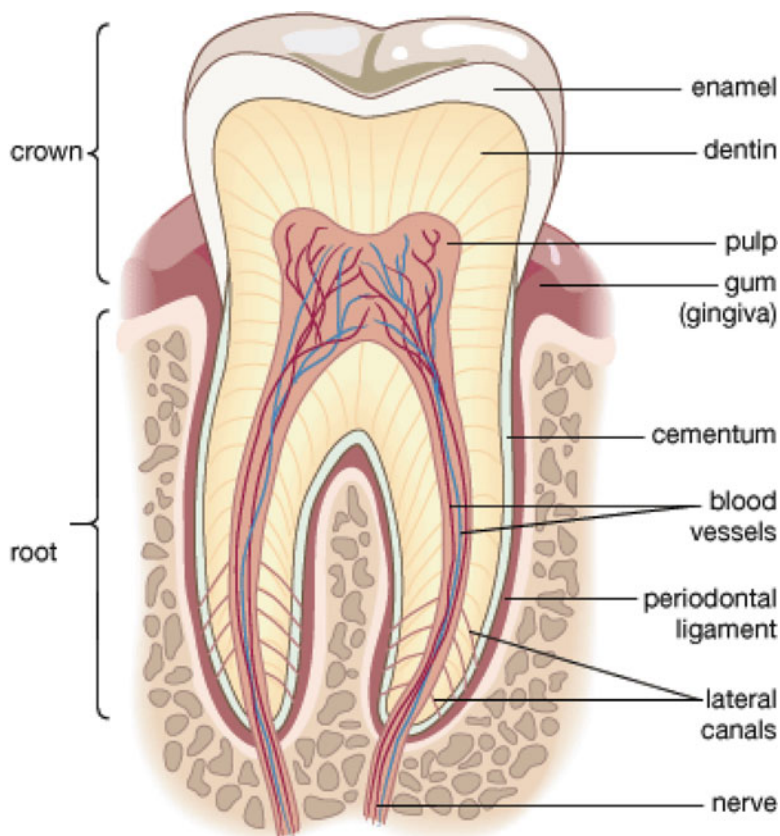
2.2 Odontoblast Differentiation

Although the main function of odontoblasts is dentin production, initiation of innate immune responses and suspected pain transmission are also roles attributed to them (Allard et al. 2006; Farges et al. 2009).

Odontoblast's terminal differentiation occurs according to tooth-specific patterns. In the Swiss mouse molar, the terminal differentiation of odontoblasts starts at the tip of the principal cusps and progressively continues through the apical parts. The specific temporo-spatial pattern of odontoblast terminal differentiation has been characterized by Ruch et al. in the following steps: (1) pre-odontoblasts withdraw from the cell cycle. (2) After the last cell division, the daughter cell that is in contact with the basement membrane elongates and polarizes. (3) These cells start to synthesize first predentin and then dentin components (Ruch et al. 1976, 1982, 1983, 1995; Ruch 1985). All these steps are completed within 6 h in the mouse (Lesot et al. 2001).

Brief descriptions follow:

1. Shortly after the cells of the IDE (pre-ameloblasts) at the sites of the future cuspal tips stop dividing and assume a columnar shape (pre-ameloblasts), the most peripheral cells of the dental papilla enlarge and become organized along the basement membrane (BM). Those cells now become pre-odontoblasts that align as a tall single layer at the periphery of dental papilla adjacent to IDE. Between pre-odontoblasts and pre-ameloblasts, BM exists as the tooth's epithelial-mesenchymal interface. IDE controls those stages and BM plays a major role as a reservoir of paracrine molecules for the continuous reciprocal epithelial-mesenchymal



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Fig. 2.3 The main structures of the tooth (<http://www.britannica.com/EBchecked/media/112882/Cross-section-of-an-adult-human-molar>)

interactions (Ruch et al. 1982; Timpl and Brown 1996). Since isolated dental papillae never give rise to differentiated odontoblasts alone, the specific signals from BM are necessary to initiate odontoblast differentiation (Karcher-Djuricic et al. 1978). Type IV collagen, fibronectin, tenascin, laminin, nidogen, hyaluronic acid, and heparan sulfate are the main components of BM (Lesot et al. 1981). Of note, in mature pulp, BMs are located at the cell-connective tissue interfaces of endothelial cells and Schwann cells (Okiji 2002).

2. From dental lamina formation to the appearance of the first postmitotic odontoblasts, 14 or 15 cell cycles may occur. After a significant lengthening of the duration of the pre-odontoblast cell cycle from approximately 10–14 h, the last cell division occurs and it is asymmetric. The mitotic spindle is oriented perpendicular to the BM during the last cell division, and at the end of the division, only the daughter cell that is in contact with the BM will give a terminally differentiated odontoblast (Fig. 2.4) (Ruch et al. 1982).

- Initiation of odontogenesis by the preodontoblasts (PO)
- Progressive addition of collagens, glycoproteins, and proteoglycans to the BL gives rise to stage specific basement membranes (BM)
- BMx might control the orientation of the spindle of PO. The daughter cells become potential mature preodontoblasts (MPO). Only the MPO in relation with the BM will overtly differentiate
- MPO controls the turn-over of proteoglycans synthesized by the preameloblasts (PA): BMx becomes BMy
- GAG modifications might increase the adenylate cyclase activity of MPO which become post-mitotic (tetraploid?) odontoblasts (PMO). These cells no longer synthesize collagen type III; fibronectin is redistributed. BMy becomes BMz
- BMz modifies the activity of the cytoskeleton: PMO become polarized odontoblasts (PoO). PoO present amplification of collagen type I and type I trimer syntheses
- The predentin (PD) secreted by functional odontoblasts (FO) maintain their functional state. The BL disappears, PA become postmitotic ameloblasts (PMA)

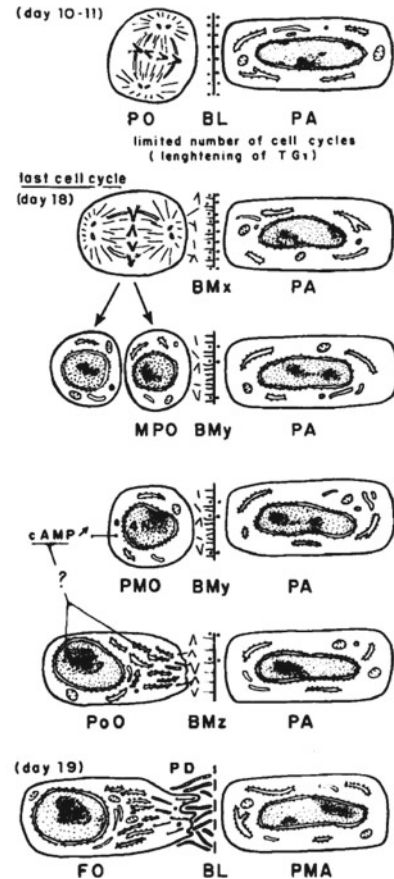


Fig. 2.4 Hypothesis concerning odontoblast differentiation (reproduced from Ruch et al. 1982 with the permission of the publisher)

3. This cell rapidly polarizes as the nucleus takes up an eccentric basal position and shows active protein synthesizing properties (Ruch et al. 1982; Ruch 1985, 1998).

Odontoblasts are post-mitotic cells and their differentiation has many properties in common with the events seen in asymmetric cell division of stem cells (Horvitz and Herskowitz 1992). For stem cells to self-renew, it has been suggested that an intrinsically asymmetric cell division whereby stem cells segregate cell fate determinants into only one of the two daughter cells by two different mechanisms occurs (Fig. 2.5) (Knoblich 2008).

In the niche-dependent type of asymmetric cell division, the mitotic spindle of dividing stem cells orients perpendicularly to the niche surface; hence it ensures that only one daughter cell can maintain contact with the stem cell niche and retain the ability to self-renew (Li and Xie 2005). Alternatively, regulators of self-renewal are localized asymmetrically during mitosis by an intrinsic mechanism, so that those

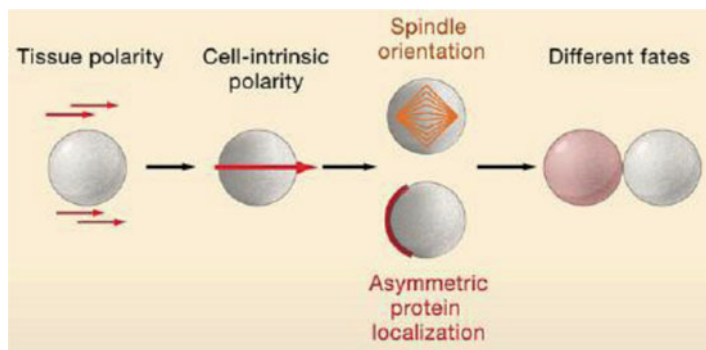


Fig. 2.5 An asymmetric cell division is defined as any division that gives rise to two sister cells that have different fates—a feature that can be recognized by differences in size, morphology, gene expression pattern, or the number of subsequent cell divisions undergone by the two daughter cells (reproduced from Knoblich 2008 with the permission of publisher)

regulators are inherited by only one of the two daughter cells (Yu et al. 2006a). In this sense, the mitotic spindle orientation during primary odontoblast differentiation may become a starting point to chase the stem cells in dental pulp. Obviously there is an asymmetrical division, in which mitotic spindle orientation occurs after withdrawal of the pre-odontoblast from the cell cycle. During the last division, the mitotic spindle, originally parallel to the BM, realigns to be perpendicular to BM and only the cell in contact with the BM is able to enter into the terminal differentiation process and become a fully differentiated odontoblast (Osman and Ruch 1976). The other daughter cell, which is not in contact with the BM, becomes a part of Höhl's cell layer. In 1896, the German scientist Höhl gave his name to those cells and Höhl cells have been assumed to differentiate into odontoblastoid cells when original odontoblasts have died (Höhl 1896). In odontoblast terminal differentiation, BM may act as a niche and after asymmetrical division of the pre-odontoblast, the Höhl cell would continue as a "stem cell" of the dental papilla and reside in the subodontoblastic layer or migrate into deeper pulp, while the other would differentiate into odontoblast.

2.3 Dental Papilla and Follicle

Simultaneous to odontoblast differentiation, IDE differentiates to ameloblasts that secrete enamel just before the first mantle layer of dentin is formed by odontoblasts. It has been thought that the proteins or growth factors secreted by ameloblasts have some effects on the terminal differentiation of odontoblasts, possibly by interacting with components of BM (Nanci 2008). When dentin has formed, the enamel producing cells assemble as a layer. Then, ameloblasts move away from the dentin leaving secreted enamel behind. Differentiating

odontoblasts need signals from differentiating ameloblasts and vice versa, meaning that tooth development needs reciprocal, complex epithelial-mesenchymal interactions (Ruch et al. 1976).

When the first calcified matrix appears at the tip of the principal cusp, the dental papilla is referred as the tooth pulp. The cells of the pulp in this stage are undifferentiated mesenchymal cells and a few collagen fibrils are seen in the extracellular matrix (Nanci 2008). The blood vessels in the dental papilla form clusters, whose position coincides with the root formation positioning. Unfortunately, there is no detailed information about angiogenesis during tooth development. On the other hand, the first nerve fibrils, as well as the vessels, approach the developing tooth during the bud-to-cap transition stage. Nerve fibrils penetrate the papilla when dentinogenesis begins. It has been assumed that the initial innervations is involved in the sensory innervation of future periodontal ligament and pulp (Nanci 2008).

The root is formed via *Hertwig's epithelial root sheet*, which consists of epithelial cells of the IDE and ODE. This sheath extends around the dental pulp and is almost closed except for the little opening, *apical foramen*, in the apical portion of the root. As root formation proceeds, epithelial cells influence the differentiation of odontoblasts from the ectomesenchymal cells at the periphery of the dental papilla as well as cementoblasts from follicle mesenchyme. This leads to the deposition of root dentin and cementum, respectively.

Although this describes the formation of a single root, multi-rooted teeth are formed in the same manner (D'Souza 2002; Nanci 2008). While roots are forming, the supporting tissues of the tooth from the dental follicle also develop. The dental follicle gives rise to various components of the periodontium, namely the periodontal ligament fibroblasts, the alveolar bone of the tooth socket, and the cementum. These structures also play a role during tooth eruption, which marks the end phase of odontogenesis (D'Souza 2002).

2.4 Formation of Permanent Dentition

Permanent dentition has the same pattern as primary dentition and the tooth germs of permanent incisors, canines, and premolars also arise from the dental lamina. However, permanent molars have no deciduous predecessors. Their dental lamina forms posteriorly beneath the lining of the epithelium of oral mucosa into the ectomesenchyme. Albeit at different times, permanent molars form in essentially the same manner of deciduous teeth. While primary dentition takes place between the sixth and eighth weeks of embryonic development, permanent dentition occurs between the 20th week in utero and tenth month after birth, and the permanent first molars between the 20th week in utero and the third molar in the fifth year of life (Nanci 2008).

In conclusion, the development of a tooth can be summarized in Fig. 2.6 (Nanci 2008).

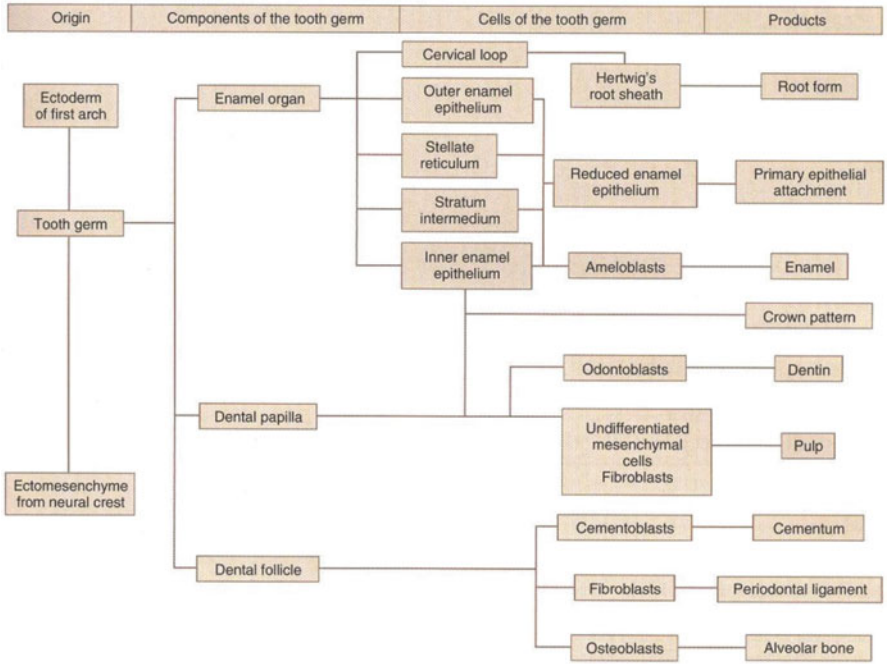


Fig. 2.6 Summary of tooth formation (reproduced from Nanci 2008 with the permission of the publisher)

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Chapter 3

Dental Pulp Is a Connective Tissue

Although dental pulp has been classified as a loose connective tissue, several unique properties such as the presence of odontoblasts, absence of histamine-releasing mast cells, tissue confinement in a hard cavity with little collateral circulation, and vascular access limited to the root apex are the features that distinguish pulp tissue from other connective tissues (Dummett and Kopel 2002).

3.1 Histology of the Dental Pulp

While dental pulp displays a characteristic arrangement in the peripheral portion, in the central core of the pulp, the basic components are arranged in a manner similar to that found in other loose connective tissues.

A single layer of odontoblasts forms a lining at the periphery of the pulp tissue. While the cell bodies of odontoblasts stay in the pulp, the long cytoplasmic processes extend into the dentinal tubules. This specific orientation of odontoblasts results in dentin and pulp acting together as a whole organ. This is called the “dentin–pulp complex.” The anatomical, developmental, and functional relationships of dentin and pulp make pulpal connective tissue responsive to dental injuries, even when it is not directly stimulated (Okiji 2002).

Subjacent to the odontoblast layer, a cell-free zone or zone of Weil is seen. This area consists of a rich network of mostly unmyelinated nerve fibers, blood capillaries, and processes of fibroblasts.

3.2 Extracellular Matrix of Dental Pulp

Dental pulp is classified as a loose connective tissue, which is rich in ground substance and contains relatively fewer fibers. The extracellular matrix is a major constituent of connective tissue and includes fibrillar proteins and ground substance. While

collagen and elastin constitute structural fibers, fibronectin and laminin are the main adhesive glycoproteins, whose primary function is to mediate cell–matrix interactions. The ground substance of the pulp is mainly composed of proteoglycans macromolecules, which consist of a protein core and varying numbers of glycosaminoglycan side chains. Hyaluronic acid, dermatan sulfate, and chondroitin sulfate are the most important glycosaminoglycans in the pulp (Okiji 2002).

Collagen type I is the most abundant collagen form designated as collagen fibers in dental pulp. While the other fibrillar collagens, types III, V, VI, and XI, are seen in the pulp, non-fibrillary type II collagen does not occur. The composition of collagen types in dentin and predentin differs considerably from that of pulp. Since the majority of collagen molecules are produced by odontoblasts, dentin collagen might not be a combined product of odontoblasts and pulp fibroblasts (Jontell et al. 1998; Okiji 2002).

A multifunctional stromal glycoprotein fibronectin is ubiquitously distributed in the pulp. It could be localized in the pulp proper as reticular networks fibrils, and in the odontoblast layer. Several roles have been attributed to fibronectin including the proliferation, differentiation, and organization of odontoblasts (Lesot et al. 2001; Zhang et al. 2007; Zarrabi et al. 2011). Elastin is always found in the pulp, as it is associated with larger blood vessels.

3.3 Cells of the Dental Pulp

Physiologically, dental pulp has many functions. Mainly they are inductive, formative, protective, and sensory functions. The postnatal dental pulp contains heterogeneous cell populations in order to maintain, defense, and repair the tissue structure.

3.3.1 *Odontoblasts*

Fully differentiated odontoblasts are 50–60 μm long columnar cells. Cell body and organelle distributions show polarization including one odontoblastic process containing a well-developed cytoskeleton and both exocytotic and endocytotic vesicles (Sasaki and Garant 1996). Terminally differentiated odontoblasts secrete the first layer of dentin (mantle dentin) and begin to move toward the center of dental papilla. During this movement, odontoblasts leave their process behind while forming into the dentin matrix. The dentin matrix mineralizes around the process and dentinal tubules forms. Dentin is constituted of thousands of dentinal tubules that cross over the dentin from the dentinoenamel junction to the pulp. Although odontoblasts display an epithelial appearance by their alignment at the periphery of pulp, the tight junctions between them play roles in cell polarization and differentiation processes rather than to permeability functions (Arana-Chavez and Massa 2004; Joao and Arana-Chavez 2004).

Other than mantle dentin, the differentiated odontoblasts continue to deposit different forms of dentins: circumpulpal, intertubular, and peritubular dentins. They are all formed up until the completion of root development and they are defined as *primary dentin*. However, odontoblasts deposit *secondary dentin* throughout life. While the secondary dentin secretion rate is slower than that of primary dentin, their structures are similar.

As stated before, odontoblasts are highly specialized, post-mitotic NC-derived cells. They produce the collagens and proteoglycans of the organic matrix of predentin and dentin. Odontoblasts also synthesize a variety of noncollagenous proteins, such as bone sialoprotein (BSP), dentin sialoprotein (DSP), dentin phosphoprotein (DPP), dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1), phosphoryn, osteocalcin, osteonectin, and osteopontin (OPN). DSPP, DSP, DPP, and DMP-1 are considered as being dentin-specific markers; however, they can be found in several other tissues with different roles. Hence, it has been shown that DSPP is not a tooth-specific protein and that dramatically different regulatory mechanisms governing DSPP expression are involved in tooth and bone (Qin et al. 2003; Suzuki et al. 2012). While DMP1 mutations have recently been shown to cause autosomal recessive hypophosphatemic rickets, tooth abnormalities have so far not been described in humans (Koshida et al. 2010). DSPP affects the mineralization of dentin more profoundly than DMP-1, whereas DMP-1 significantly affects bone mineralization and importantly controls serum phosphate levels (Suzuki et al. 2012). Although small integrin-binding ligands, the N-linked glycoproteins (SIBLINGs) group of extracellular matrix proteins involved in bone and teeth mineralization (family members include MEPE, DMP1, OPN, BSP, enamel, DSPP, and statherin) are not currently regarded as being specific for dentin alone (Hao et al. 2005; Simon et al. 2009). Moreover there is limited information regarding the distinguishable biochemical features of bone and dentin.

3.3.2 Fibroblasts

Although there is detailed information about odontoblast differentiation during odontogenesis, little or no attention has been placed on the differentiation of the other cells of dental pulp. Fibroblasts are the predominant cell type in dental pulp. They are mostly found in the cell-rich zone of the pulp, which is localized beneath the subodontoblastic layer. Their particular role is maintaining the pulp matrix. They synthesize mainly type I and type III collagens, and a wide range of noncollagenous extracellular matrix components, such as proteoglycans and fibronectin. In the adult pulp, fibroblasts appear as flattened spindle-shaped cells. Fibroblasts are also included in the degradation of matrix components and thus are essential in the remodeling of pulp tissue (Okiji 2002).

3.3.3 *Undifferentiated Mesenchymal Cells*

Undifferentiated ectomesenchymal cells have been traditionally emphasized in dental literature. According to Nanci:

They are found throughout the cell-rich area and the pulp core and often are related to blood vessels. Under the light microscope, undifferentiated mesenchymal cells appear as large polyhedral cells possessing a large, lightly stained, centrally placed nucleus (Nanci 2008).

Since there is no known marker(s) for such an undifferentiated mesenchymal cell population, it is still unclear if dental pulp stem cells are part of primitive cell populations, which may be shown by undifferentiated mesenchymal cells.

3.3.4 *Immunocompetent Cells*

Immunocompetent cells are normal residents in the connective tissue of the pulp and they respond to the situations that threaten tooth integrity, such as caries, tooth fracture, and cavity preparation. The human tooth is the target of a substantial number of oral bacterial agents that initiate the development of carious lesions (Okiji 2002). In the development of caries following enamel demineralization, dentin becomes exposed to the oral environment. This enables cariogenic bacteria to cause inflammation and subsequent immune system responses in the underlying dental pulp through the diffusion of by-products into dentin tubules (Farges et al. 2009). Dental pulp can also respond immunogenically to cavity preparation and restoration, but these immune responses are different to those caused by caries conditions (Yoshida et al. 2003). Pulp retains its defense capacity even in aged pulps (Kawagishi et al. 2006).

Inflammatory and immune system cells, such as dendritic cells, neutrophils, histiocytes/macrophages, and T/B lymphocytes, are included in pulp. While B lymphocytes differentiate into antibody-secreting plasma cells (humoral immunity), T lymphocytes play the main part in specific immune responses. Cytotoxic T lymphocytes (CD8⁺) cause lysis of infected cells and helper T lymphocytes (CD4⁺) produce cytokines to orchestrate immunity. CD8⁺ cells bind Class I major histocompatibility complex (MHC) molecules in humans very polymorphically. These are designated as human leukocyte antigens, HLA, -A, and -C. HLA class I molecules are expressed in almost all cells of the body. Class II MHC molecules, on the other hand, bind to CD4⁺ molecules on T lymphocytes and they are HLA-DR, -DP, and -DQ in humans. In contrast to HLA type I, type II molecules are expressed in limited types of cells, mainly on dendrites and B lymphocytes. In intact teeth, they are distributed mainly in and around the layer of odontoblasts with dendritic profiles and are called pulpal dendritic cells (Shackelford et al. 1982; Okiji 2002).

Ohshima et al. searched for HLA-DR-immunopositive cells in human dental pulp. They found them densely distributed throughout the pulp as spindle-like

or dendritic in profile. At the periphery of the pulp tissue, the HLA-DR-immunopositive cells were predominantly situated in the subodontoblastic layer, with some located in the odontoblast layer and/or predentin. These results clearly indicate that besides their roles in the defense reactions against injury, the HLA-DR-immunopositive cells in the odontoblast layer and/or predentin have some regulatory function on the odontoblasts under certain physiological conditions (Ohshima et al. 1999). Besides, Yoshida et al. showed the immunoreactivity for HLA-DR in Schwann cells of carious lesion. Since the immunoreactivity depended upon the severity of the carious lesion, they suggested Class II-expressing Schwann cells might function as antigen-presenting cells in addition to dendritic cells (Yoshida et al. 1998). Moreover, accumulating evidence shows that odontoblasts initiate immune/inflammatory events within the dental pulp in response to cariogenic bacteria (Durand et al. 2006; Veerayuthwilai et al. 2007; Staquet et al. 2008; Farges et al. 2009). Additionally, fibroblasts might recognize viruses present in the dental pulp (Glick et al. 1991). However, Staquet et al. suggested that odontoblasts and pulp fibroblasts differ in their innate immune responses to oral microorganisms that invade the pulp tissue (Staquet et al. 2008).

3.4 Neuronal and Vascular Networks

The complex and rich neuronal and vascular networks are important for regenerative responses. With the impressive nerve fiber density of the pulp, the nerve terminals of sensory fibers store neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP), neurokinin A, neuropeptide Y, and vasoactive intestinal polypeptide (Caviedes-Bucheli et al. 2008). Along with their roles in neurogenic inflammation, those peptides may modulate immune responses. It has been shown that pulpal sensory neurons have afferent pain detection and efferent (i.e., neurogenic inflammation, immunomodulatory, and healing) functions (Okiji 2002). While 70–90 % of the fibers are unmyelinated, recent experiments suggest that pulpal afferents originate from fibers that are myelinated at more proximal locations (Henry et al. 2012).

3.5 Deciduous (Primary) Tooth Dental Pulp

Deciduous and permanent teeth have a similar developmental process, although the former needs a shorter time to develop. The most important difference is that deciduous teeth undergo root resorption. Deciduous teeth start to erupt into the oral cavity by the sixth month after birth and shedding starts around the age of 7 years. Primary molars erupt around the age of 2.5 years and the last primary molars are shed around the age of 12 years. Hence, deciduous dentition functions for approximately 8.5 years. Their life span can be divided into periods: young, mature,

and old, after which resorption and shedding occur. The growth period and root maturation last about 4.75 years, and root resorption and exfoliation take about 3.5 years. Hence, permanent dentition has seven to eight times longer in which to function than the deciduous teeth (Avery 2002). Root resorption of deciduous teeth and concurrent root formation, alveolar bone arrangement, and eruption of the successor permanent teeth (Marks and Schroeder 1996) in a few millimeters space in the alveolar bone are fascinating, very well coordinated, and yet mysterious events of human body.

Deciduous and permanent teeth have similar basic dentinal structures. However, dentin thickness in deciduous teeth is lesser than in permanent teeth. The pulp of deciduous teeth is proportionately larger than in permanent teeth and there are many accessory canals in the root pulp (Dummett and Kopel 2002).

Clinically different pulp responses between deciduous and young permanent teeth to chemical, bacterial, or traumatic events have no counterpart histologically. Both are similar in basic histological architecture in terms of vasculature, connective tissue, and odontoblastic and subodontoblastic zones (Avery 2002). It has been noted that the presence of a cap-like zone of reticular and collagenous fibers in the primary coronal pulp is the only differences between deciduous pulp tissue and young permanent pulp tissue (Fox and Heeley 1980). Anatomically deciduous roots have an enlarged apical foramen, in contrast to the constricted foramen of permanent roots. Despite their abundant blood supply, deciduous teeth demonstrate exaggerated inflammatory responses, and internal and external root resorption can be seen more often than in permanent teeth: the greater the inflammation, the more severe the resorption (Dummett and Kopel 2002). During eruption and physiological resorption, pulp tissue reciprocates inflammatory events by unknown mechanisms. In permanent dentition root resorption is a dental complication that occurs as a result of severe infection or traumatic events. Except the inflammation-triggered ones, root resorption is usually free of pain until the advanced stages. As discussed, deciduous teeth root resorption is a completely natural process, which allows exfoliation of the primary teeth. The observed inflammatory cell infiltration due to resorbing the apex (in deciduous teeth resorption process) is an interesting phenomenon (Fig. 3.1). It has been shown that immunocompetent cells of deciduous tooth pulp increase in number along with the progress of physiological root resorption (Angelova et al. 2004; Simsek and Duruturk 2005). However, the normal structure of the coronal pulp is preserved even until the tooth exfoliated (Furseth 1968; Simsek and Duruturk 2005), suggesting deciduous dental pulp might have an immune-modulatory coronal pulp and resorbing apical parts. Likewise, it has been suggested that impaired accumulation of T cells within the decidua might be related to mechanisms of feto-maternal immune tolerance (Nancy et al. 2012). Whether such a mechanism exists, including possible epigenetic silencing of key T-cells attracting inflammatory chemokine genes (Nancy et al. 2012) in deciduous dental pulp cells, and related regulation of immune tolerance of dental pulp stem cells in deciduous teeth modulating immune responses by secreting cytokines and key transcriptional factors (Yildirim et al. 2008), warrants further investigations.

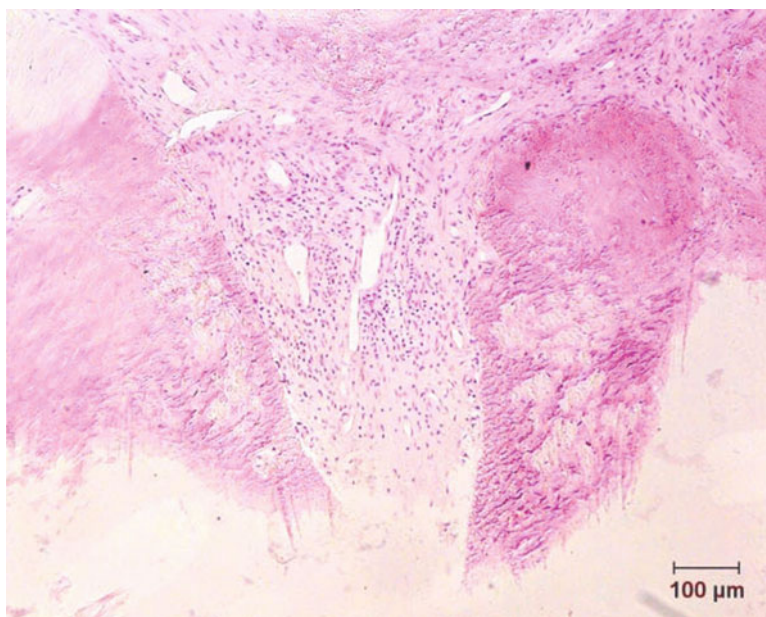


Fig. 3.1 A dense inflammatory reaction is seen throughout the apex of the resorbing deciduous tooth (H&E, bar 100 μ m)

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Chapter 4

Dental Pulp Stem Cells (DPSC)

Stem cell technology is developing at a rapid pace. Every piece of seemingly unrelated, floating data is being conglomerated to broaden current knowledge and move closer to the inevitable shift in paradigms of current fundamental cell biology systems. The question of what a stem cell actually is may no longer require further discussion; however, there are still some differences in opinion between groups in terms of their functional roles.

4.1 Evidence for Dental Pulp Stem Cells: Emergence of a New Generation of Odontoblast-Like Cells

The dentin–pulp complex has certain intrinsic healing capacities. Reparative dentin is formed in response to external factors effecting the pulp, thus stimulating the dentinogenic progenitors that are responsible for dentin repair, contained in dental pulp (Rutherford et al. 1993, 1994; Mao et al. 2006; Yildirim et al. 2011b). The mechanisms for reparative dentinogenesis have been discussed extensively in the literature and recent studies have further enriched our understanding of dental pulp and dentin regeneration (Schroder 1985; Mullane et al. 2008; Huang 2009; Kim et al. 2010a; Mao et al. 2012). However, the relationship between different characteristics of dental pulp, substantial infection or trauma of dental pulp, and intrinsic healing of dental pulp is not well understood.

Odontoblasts, as post-mitotic cells, remain in a tooth for its lifetime, secreting secondary dentin, though their synthesizing functions decrease in time (Arana-Chavez and Massa 2004). Moreover, odontoblasts are also responsible for the secretion of a *tertiary dentin* in response to caries or any other mechanical or chemical problems.

Primary odontoblasts secrete dentin as secondary dentin at a very slow rate. Conditions such as tooth decay, attrition and incidental or intentional trauma and microleakage between restoration and dentin stimulate the odontoblasts and their defense and/or repair responses. Several types of injurious agents can stimulate odontoblasts: bacteria and their toxins (lipopolysaccharides and other bacterial

toxins), $\text{TNF}\alpha$ (via p38 phosphorylation of the MAPK pathway), and the liberation of cytokines and growth factors during demineralization of the dentin matrix by caries (McLachlan et al. 2003; Durand et al. 2006; Paula-Silva et al. 2009). Once stimulated, odontoblasts enter an active state and secrete the reactionary dentin (Smith et al. 1995). However, if the nature, magnitude, or duration of the problem causes irreversible damage or the dental pulp is exposed to the oral cavity by trauma or dental procedures, primary odontoblasts die. Since the odontoblasts are post-mitotic terminally differentiated cells, they cannot proliferate to replace irreversibly injured odontoblasts. Therefore, pulpal regenerative processes take place to initiate reparative dentinogenesis and pulp progenitor/stem cells differentiate into odontoblast-like cells, which secrete reparative dentin. In this way, reactionary and reparative dentins are the constituents of tertiary dentin. While reactionary dentin is laid down by the surviving preexisting odontoblasts, reparative dentin can be produced by recruited secondary odontoblast-like cells (Smith et al. 1995).

4.2 Origin of Progenitor Cells

The suggestion that the replacement of irreversibly injured odontoblasts by predetermined odontoblastoid cells that do not replicate their DNA after induction (Höhl 1896) was rejected by auto-radiographic studies using tritiated thymidine (3HTdR or [^3H]-thymidine) (Feit et al. 1970; Fitzgerald 1979). Metabolic incorporation of [^3H]-thymidine into cellular DNA is still a widely used protocol to monitor rates of DNA synthesis and cell proliferation (Hu et al. 2002). By this mean, the recruitment and induction of cell populations and DNA replication cycles before differentiation into functioning odontoblasts have been shown by several researchers (Yamamura et al. 1980; Yamamura 1985; Fitzgerald et al. 1990; Tziafas and Kolokuris 1990).

Yamamura et al. suggested that:

Injury caused by partial pulpotomy capped with pure calcium hydroxide leads either to dedifferentiation of mesenchymal cells in the deep pulp into undifferentiated mesenchymal cells, which then migrate toward the wound, undergo all phases of division and subsequently redifferentiate into new odontoblasts, or to direct induction and differentiation of existing undifferentiated mesenchymal cells (Yamamura 1985).

Fitzgerald et al. showed that after pulp exposure in primate teeth, there was a significant cellular influx from pulp proper to the wound site and repair of the wound by secreting a dentin-like matrix as odontoblasts do (Fitzgerald et al. 1990). They suggested that those cells might be derived from a population of pulpal cells and perivascular tissues that reside in the deeper sites of the pulp. Additional to labeled odontoblast-like cells, they have also observed unlabeled odontoblast-like cells at the exposure site. Since “no label” indicates “no DNA replication,” the authors rationally exclude the possibility of G_2 -blocked, predetermined cells as a source of those unlabeled odontoblast-like cells. Instead, they suggested that those unlabeled odontoblast-like cells had replicated their DNA before the tritiated thymidine injection in those

cells. Therefore, the authors concluded there is either the presence of a synchronous induction of cells that then undergo synchronous differentiation, or a synchronous induction of heterogeneous population of cells (Fitzgerald et al. 1990).

These seminal papers might give clear ideas about the presence of dental stem cells in pulp proper and obviously tritiated thymidine labeling was a smart tracer for their niche. Currently, perivascular cells (pericytes), undifferentiated mesenchymal/mesectodermal cells, fibroblasts or Höhl's cells of sub-odontoblastic layer are proposed dental pulp progenitors. However, controversy still exists about the origin and precise identification of those progenitor or stem cells, because of the usage of different terminology (progenitor versus stem cells) and different experimental conditions that the data derived (in vivo versus in vitro). The niche concept will be discussed further in the related section.

4.3 Dental Pulp Stem Cells (DPSC)

Adult stem cells are undifferentiated cells, which divide to replenish dying cells and regenerate damaged tissues, and found in numerous tissues throughout the body (Anderson et al. 2001). To date, other than bone marrow stem cells, mesenchymal stem cells (MSC) have been identified in a variety of tissues, such as adipose tissue, peripheral blood, spleen, brain, synovial fluid, dermis, muscle, dental pulp, umbilical cord, skin, cornea, retina, liver, pancreas, and intestines (Prockop 1997; Rodriguez et al. 2005). However, there are significant differences in their proliferation and differentiation abilities, and in harvesting procedures among these MSC (Guilak et al. 2004). One of the major obstacles in stem cell research is the difficulty in isolating them due to the lack of universally accepted markers for these cells.

The well-known regenerative potential of dental pulp has indicated the existence of stem cells for decades. Finally, in 2000 Gronthos et al. isolated a population of cells that are highly proliferative, and display a hierarchy for cellular differentiation and multipotentiality (Gronthos et al. 2000). Since dental pulp is derived from mesectoderm or ectomesenchyme, it has been shown that the clonogenicity, self-renewal and proliferation capacities, expression pattern of specific markers, and multipotency of DPSC display comparative equivalence with NC cells (Sasaki et al. 2008; Kerkis and Caplan 2012).

In general DPSC show the common properties of adult stem cells. In 2007, The International Society for Cellular Therapy (ISCT) agreed that an MSC should adhere to plastic in standard culture conditions, express ($\geq 95\%$) CD105, CD73, CD90 and not express ($\leq 2\%$) CD45, CD34, CD14 or CD11b, CD79 α or CD19, HLA-DR, and finally should give at least three differentiated lineages: osteoblastic, adipogenic, and chondroblastic (which needs to be demonstrated by staining of in vitro differentiated cell cultures) (Dominici et al. 2006). Reports from the last 30 years show many examples for the “new” stem cell lineages that completely satisfy these criteria. Although DPSC have been characterized by multi-potent differentiation, the expression of some stem cell markers, dentin regeneration in vivo,

and colony-forming ability in culture, they also contain heterogeneous populations of cells from the pulp (Gronthos et al. 2000, 2002; Liu et al. 2006).

DPSC can

- Promote the proliferation and differentiation of neural cells in the hippocampus of mice (Huang et al. 2008a)
- Prevent the progression of liver fibrosis and contribute to the restoration of liver function in rats (Ikeda et al. 2008)
- Reduce the area of myocardial infarction, improve ventricular function, and induce the revascularization by intra-cardiac injection (Gandia et al. 2008)
- Reconstruct the corneal epithelium in a model of total limbal stem cell deficiency (Gomes et al. 2010)
- Form bone when combined with platelet-rich plasma or hydroxyapatite (Yamada et al. 2010)
- Repair critical-sized calvarial defects in immunocompromised mice (Huang et al. 2006; Yamada et al. 2010)
- Engraft and stimulate angiogenesis and vasculogenesis in models of hind limb ischemia in mice (Iohara et al. 2008)
- Contribute to developing the embryos of mice (Siqueira da Fonseca et al. 2009)
- Migrate, engraft, and display myogenic potential when injected into dogs (golden retriever) with muscular dystrophy (Kerkis et al. 2008)
- Generate tooth structures (Yildirim et al. 2011a)

4.4 DPSC from Deciduous Teeth Pulp

Since exfoliation of deciduous teeth is a physiological phenomenon and every child has 20 deciduous teeth, collecting them for isolation of adult stem cells from their pulpal remnants offers an easy, noninvasive, and ethically free method. On the other hand, dental pulp of permanent teeth from elderly patient has certain limitations for stem cell isolations, since the presence of subpopulations of stem cells may be greatly restricted in aged pulp cells. Because of the deleterious effects of old niches on young cells and acquired alterations in self-antigens, stem cell regenerative capacity within aged niches has been questioned (Hasler and Zouali 2005; Carlson and Conboy 2007; O'Connor et al. 2009).

As a matter of fact, stem cells from human exfoliated deciduous tooth pulp has been isolated and named as SHED (Stem cells from Human Exfoliated Deciduous teeth) in 2003 (Miura et al. 2003). They are highly proliferative postnatal stem cells capable of differentiating into odontoblasts, adipocytes, neural cells, and osteo-inductive cells (Miura et al. 2003). SHED was shown to be capable of generating a tissue that closely resembles human dental pulp (Cordeiro et al. 2008). It has been reported recently that SHED contains a higher proportion of the side population than DPSC (Wang et al. 2010). Moreover SHED have the potential to differentiate

into functional vascular endothelial cells (Cordeiro et al. 2008; Sakai et al. 2010), can promote bone formation in immunocompromised mice (Miura et al. 2003), and give better osteogenic response to retinoic acid treatment than periodontal ligament stem cells (Chadipiralla et al. 2010).

Although some minor discrepancies regarding cell surface antigen profiles, and differentiation potentials into osteogenic, adipogenic, and chondrogenic lineages have been reported so far (Huang et al. 2009; Nakamura et al. 2009; Govindasamy et al. 2010; Kerkis and Caplan 2012; Wang et al. 2012), the main differences between SHED and DPSC are in their proliferation rate and colony-forming properties, which are higher in SHED than DPSC (in parallel, the doubling time of SHED is shorter than DPSC) (Nakamura et al. 2009; Govindasamy et al. 2010; Wang et al. 2012). The other difference is the expression of pluripotency markers, which are again higher and more abundant in SHED. Many researchers have reported that SHED is a more primitive, pluripotent population of cells than DPSC, bone marrow-MSC and umbilical cord stromal stem cells (UCSC) (Kerkis et al. 2006; Nakamura et al. 2009; Govindasamy et al. 2010; Chen et al. 2012). On the other hand, it has been proposed that DPSC have a higher propensity toward neural lineage, expressing significantly higher rates than SHED (Govindasamy et al. 2010).

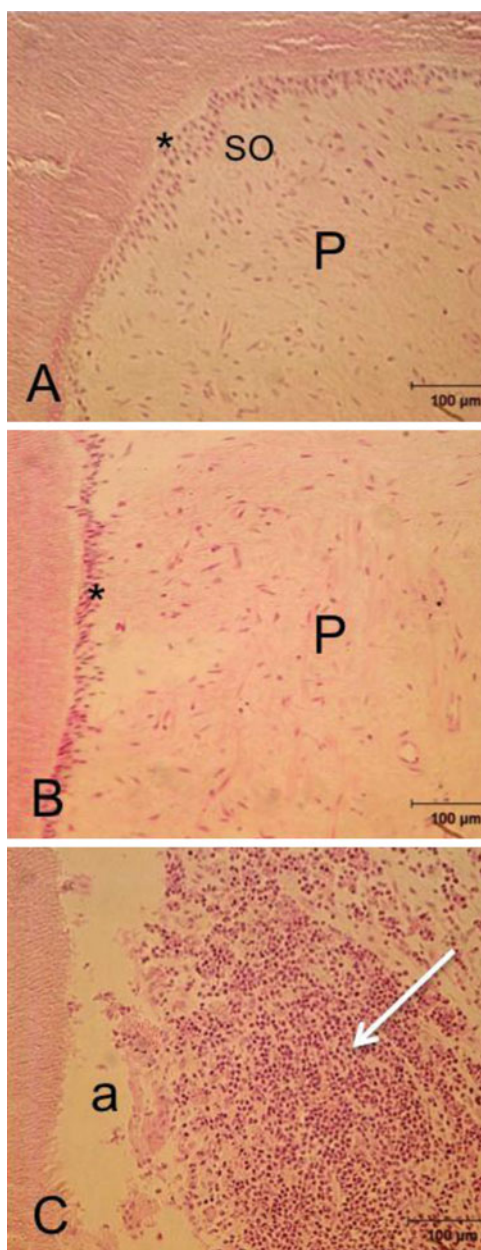
4.4.1 The Degree of Resorption of the Teeth Related to Stem Cell Isolation

The exfoliation of deciduous teeth is a physiologic phenomenon. Histological changes associated with the process of shedding in human beings have been well documented, and it has been shown that pulp tissue structure maintains its integrity till the late phase of exfoliation (Sahara et al. 1993; Sasaki 2003). Although there is an increase in the numbers of inflammatory cells in the active resorption area around the root(s) as well as mononuclear cells in the subodontoblastic area, regular odontoblasts still exist along the dentin walls (Fig. 4.1).

When roots are resorbed completely, odontoclasts appear in the coronal pulp (Fig. 4.2a, b) and they start to resorb predentin. In that phase coronal pulp contains many mononuclear and inflammatory cells (Fig. 4.2c). TRAP-positive mononuclear cells (presumed to be preodontoclasts) (Sahara 2001) can be seen on predentin where they fused to form TRAP-positive multinucleate odontoclasts that resorbed the root (Fig. 4.3).

When the resorption is completed, only minute amounts of epithelium and periodontal ligament can retain the tooth in the dental arch and resorbed sharp edges may cause pain during biting and eating. In the routine practice of pediatric dentistry we rarely extract resorbed deciduous teeth, unless the patient wants relief from the pain. In that case, with little regional or only topical anesthesia, the resorbed tooth can be separated from the periodontal ligament easily, and hence be extracted. Likewise, deciduous teeth are shed with little bleeding naturally, since the final shedding would occur by the tearing of the narrow tissue bridges by migrated gingival epithelium

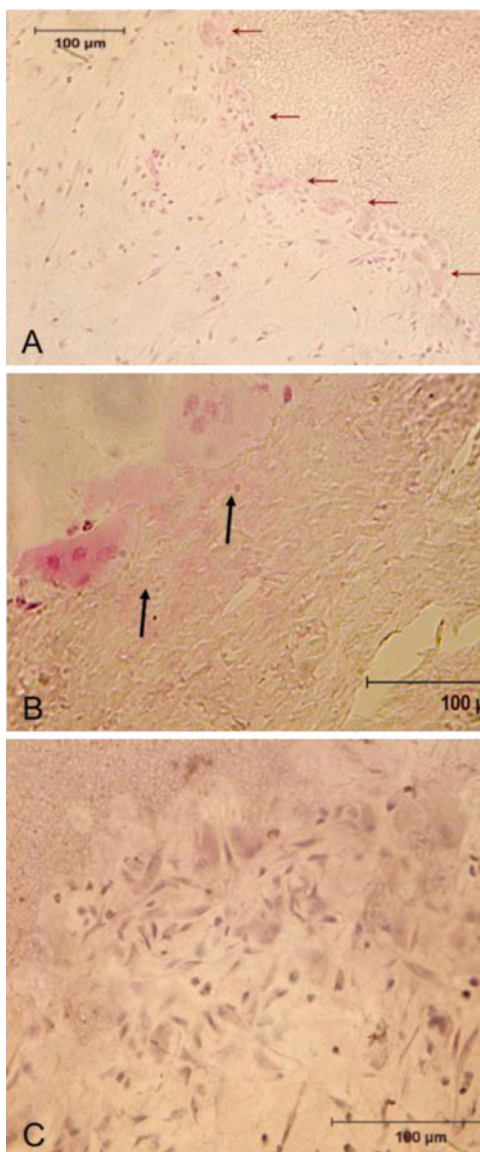
Fig. 4.1 The histological sections show healthy pulp (P) and firm layers of odontoblasts (*asterisk*) and subodontoblasts in coronal (a) and servical (b) portions, although numerous inflammatory cells invaded the resorbed root (c) (*a* artificial detachment, H&E, bars 100 μ m)



under the crown. In this final stage of resorption, there are only crowns, which their pulp chambers invaded by the oral epithelium (Fig. 4.4) (Sahara et al. 1993).

Stem cell properties of deciduous dental pulp cells have been compared with the cells obtained from exfoliated and orthodontically extracted no-resorption teeth

Fig. 4.2 In the final phase of resorption odontoclasts occur in the coronal dental pulp and start to resorb predentin (arrows in **a** and **b**). In that phase coronal pulp has more heterogeneous population including many mononuclear cells (H&E, bars 100 μ m) (**c**)



(Bernardi et al. 2011), and it has been reported that it was not possible to establish cell cultures from the teeth that did not show any visible resorption. Zhu et al. broadened the classification of resorbing teeth samples as stable, middle, and final stages and compared those samples with the non-resorbed deciduous teeth. They found that the stem cells obtained from the final resorption stage group were equal to SHED, whereas stem cells obtained from middle stage group exhibited higher

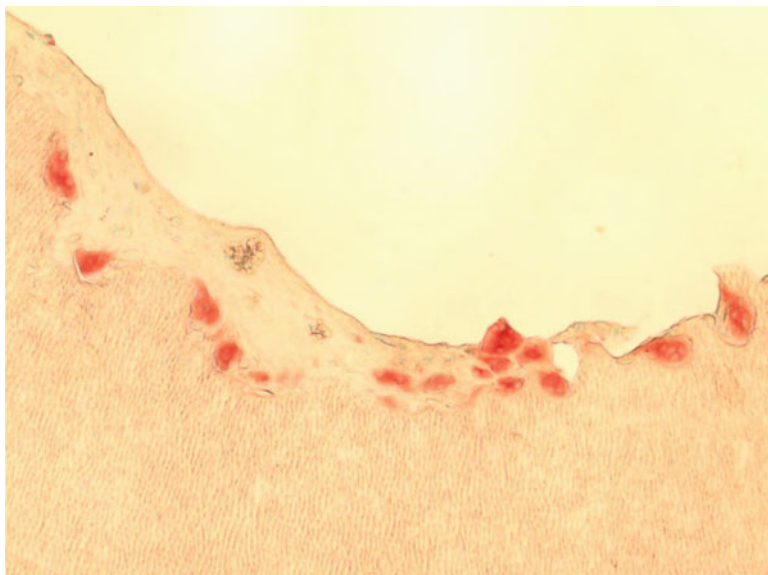


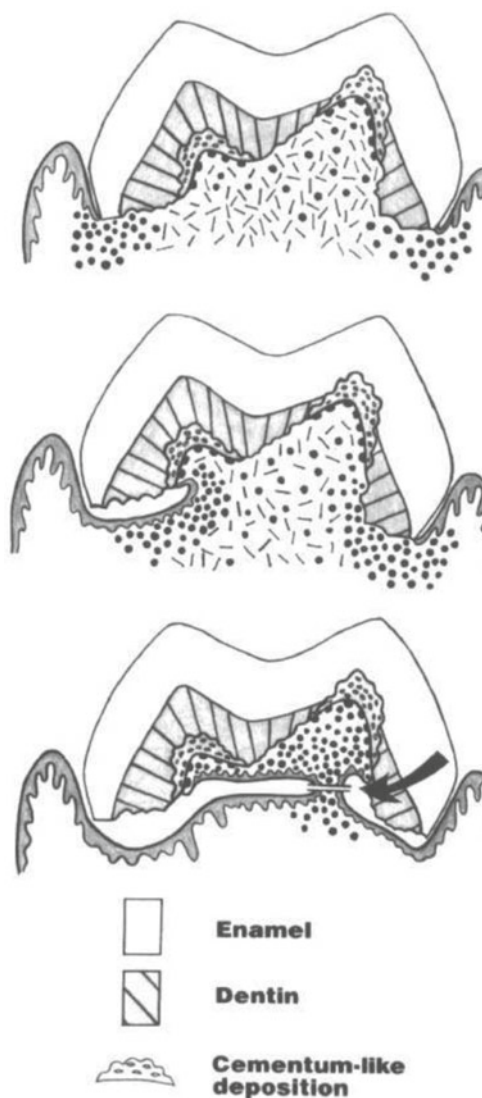
Fig. 4.3 Dark TRAP-positive multinucleate odontoclasts that reside in the lacunae on predentin (TRAP staining, 400 $\times$)

proliferation potential than those in the final stage group, yet much higher than those of the stem cells from stable resorption group (Zhu et al. 2012).

Exciting possibilities exist despite the available pulp tissue in the exfoliated deciduous tooth being extremely small for stem cell isolation. The remnants of the pulp reside only in the pulp chamber as seen by dashed lines on the crown in Fig. 4.5, and it may occupy approximately 9–13 mm³ in an upper incisor (if we consider the pulp chamber as being cylindrical with a radius of approximately 1 mm and a height between 3 and 4 mm). Since the pulp is a loose connective tissue, the weight of the tissue can be estimated. In order to compare stem cell properties of two different stromal tissues (human umbilical cord stroma and deciduous dental pulp), we isolated mesenchymal cells from 15 cm-long cords and four exfoliated deciduous teeth. At the end of P₀ (14 days) overall yield (10⁶ cells) was the same in T25 cm² flasks for both cell populations (Oktar et al. 2011).

Continuing the discussion about the increase of inflammatory molecules during resorption (Angelova et al. 2004; Simsek and Duruturk 2005), it has been suggested that cellular influx could promote the release of cytokines acting in the activating stem cells during resorption and the increased proliferation and mineralization potential of SHED would be a response to active resorption (Bernardi et al. 2011). It has been hypothesized that SHED may promote osteoclastogenesis, during the active resorption process via several cytokines including the RANKL/OPG duo, and this function may subside in the final stage, thereby protecting the teeth from excessive resorption (Yildirim et al. 2008; Bernardi et al. 2011; Zhu et al. 2012).

Fig. 4.4 Schematic representation of migration of the dento-gingival junction (DGJ) epithelium and gingival epithelium in the process of exfoliation of a human deciduous tooth. After the roots are completely resorbed, the DGJ epithelium gradually migrates toward the resorbed surface, and finally reaches the surface of the pulp chamber wall. Accompanying this migration, the gingival epithelium also proliferates and migrates toward the resorbed regions, and eventually is present under the crown. Therefore, the migrated gingival epithelium makes narrow tissue necks under the crown. The final shedding would occur by the tearing of these narrow tissue bridges (*arrow*) (reproduced from Sahara et al. 1993 with permission of the publisher)



4.5 Niche(s) in Dental Pulp

In healthy tissues, stem cells are found in their specific niche environment. The stem cell niche concept is coming from earlier work on the relationship between spleen colony forming cells and hematopoietic stem cells. Schofield proposed in 1978 that the niche is a physiologically supportive microenvironment for stem cells (Schofield 1978). Voog and Jones have defined the stem cell niche as follows:

Stem cell niches are discrete and dynamic functional domains that influence stem cell behavior to govern tissue homeostasis under diverse physiological (development and aging)

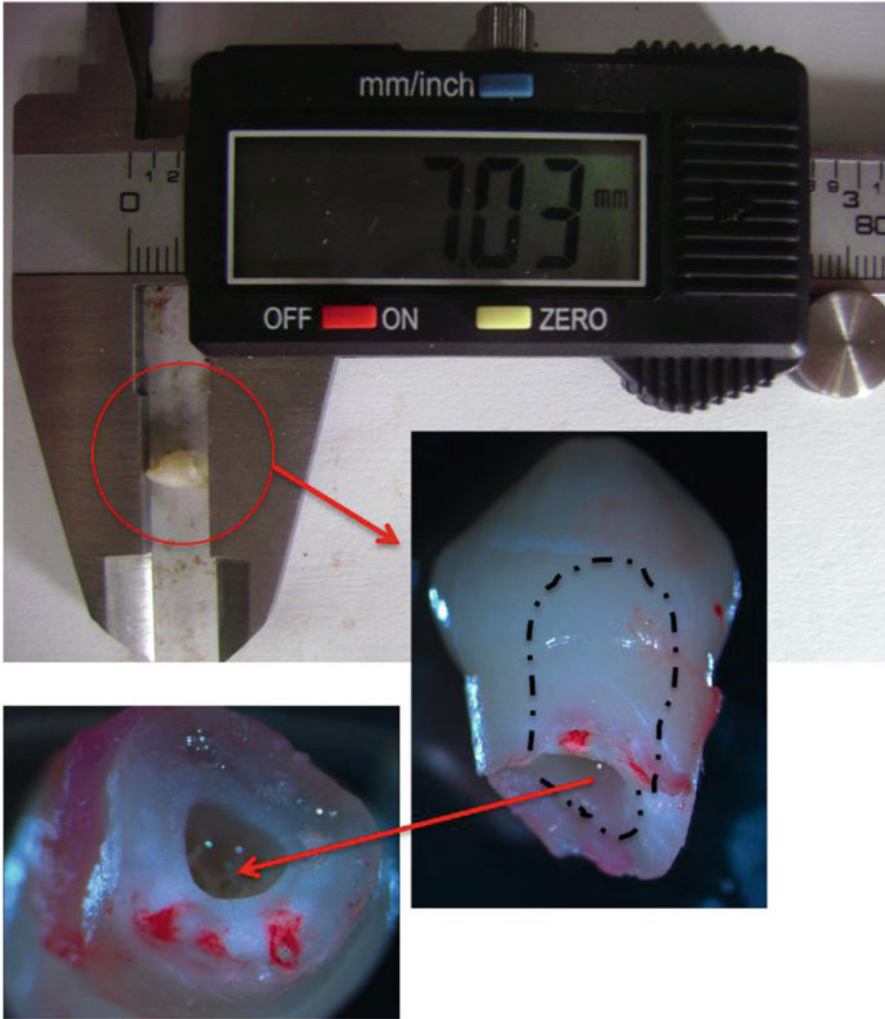


Fig. 4.5 An exfoliated deciduous tooth can be measured approximately as 7.00 mm including the crown. Pulp tissue resides in the coronal part. *Dashed lines* show the dimensions of pulp chamber

and pathological (injury and disease) conditions. The niche must be flexible in order to coordinate stem cell behavior with homeostasis and repair; however, the plasticity of a niche may be co-opted in cancer and chronic disease.

The idea that specialized environments within tissues can preserve proliferative potential and block maturation of adult stem cells was the first description of the stem cell niche hypothesis. Implicit in this model is the prediction that removal of stem cells from the niche results in loss of stem cell identity, self-renewal capacity, and the onset of differentiation. As such, the niche would provide a mechanism to precisely balance the production of stem cells and progenitor cells to maintain tissue homeostasis. Therefore, a stem cell niche is not

defined solely by the presence of stem cells but also by the ability to regulate stem cell behavior (Voog and Jones 2010).

The niche concept nestles several factors in a multidimensional space of resources. They are interactions between stem cells and neighboring differentiated cells, adhesion molecules, extracellular matrix components, the oxygen tension, growth factors, cytokines, physiochemical nature of the environment including pH, ionic strength, and metabolites (Schraufstatter et al. 2012).

Replication of a niche *in vitro* will allow predictable and controllable conditions for regenerative therapies. However, so far there are no available culturing conditions, which help to maintain the integrity of the adult stem cells over times.

Pulp tissue is mostly heterogeneous in nature. It would not be unreasonable to surmise that subodontoblastic and cell-rich zones of pulp tissue may include dental pulp stem cells, since those areas are more prone to attacks coming from dentinal tubules. On the other hand, those areas are also prone to destruction because of advanced caries. Hence, the safer place for stem cell residence would be pulp proper, especially at the periphery of main arterioles, or possibly both. Alternatively, DPSC may reside in the deeper pulp proper in a quiescent manner. When the “enter cell cycle” command arrives to those cells, they may divide asymmetrically. From the results of the tritiated thymidine labeling experiments, it can be assumed that while the labeled cells found next to the wounded area are the ones that caused odontoblast differentiation to migrate to the wound site, the other daughter cells that are the exact copies of the maternal ones may reside in this pulp proper niche for the next signal (Fitzgerald et al. 1990).

Taken together, the replacement cell population in pulp healing could be located anywhere within the coronal pulp (Fitzgerald 1979; Yamamura et al. 1980; Yamamura 1985; Tziafas and Kolokuris 1990). It has been shown that STRO-1⁺ and CD146⁺ cells are located in close proximity to blood vessels (Spath et al. 2010) and STRO-1 positive DPSC express von Willebrand factor, CD146, α -smooth muscle actin, and a pericyte-associated antigen 3G5 (Shi and Gronthos 2003). Although the reliability of STRO-1 as a stem cell marker has been questioned recently (Lin et al. 2011), the expression of these vascular antigens has been interpreted as being that these STRO-1 type stem cells might be of stem cell origin (Shi and Gronthos 2003).

To date, no specific markers for odontogenic neural crest or for the odontoblast lineage could be detected. However, the fact is that mitotic cells corresponding to stem cells are recruited in response to injury (Yamamura 1985; Tziafas and Kolokuris 1990; Goldberg et al. 2006; Harichane et al. 2011). The origins of these cells may be related to the primary odontoblasts, because during tooth development, the NC-derived cell population of the dental papilla gives rise to odontoblasts after an asymmetrical division. The other daughter cell called Höhl cell may reside in the dental pulp as a small population of stem cells within the dental pulp throughout life. BM is located at the cell-connective tissue interfaces of endothelial cells and Schwann cells in mature pulp. Schwann cells are a variety of glial cell that keep peripheral nerve fibers (both myelinated and unmyelinated) alive (Lehmann and

Hoke 2010) and endothelial cells are from endothelium, which is the thin layer of cells that lines the interior surface of blood vessels and lymphatic vessels. Endothelium forms an interface between circulating blood and lymph in the lumen and the rest of the vessel wall (Cines et al. 1998). Since the vascular and neuronal elements in dental pulp are really impressive, it is rational to assume that the “niche” of the dental pulp stem cells is around the vessels and subodontoblastic area, and that the dental pulp is able to specifically respond to inductive signals with the similar embryological process of BM-mediated induction for odontoblast differentiation (Ruch et al. 1982).

4.6 Epigenetics in Dental Pulp

An intricate signaling network that can regulate stem cell fate in the dental pulp might be useful for understanding transcriptional and epigenetic control mechanisms of the DPSC niche.

Epigenetics describes mitotically heritable modifications of DNA or chromatin without altering the nucleotide sequence. DNA methylation, histone modification, histone variants, and noncoding RNAs are mechanisms of epigenetic regulation (Bird 2002). Dental pulp is a completely dynamic tissue in constant dialogue with the environment. Teeth, and therefore dental pulp is under the threat of bacterial and viral attacks due to caries, traumatic events, extreme heat (as a result of the hot or cold consumption), teeth grinding, dentin sensitivity, oral habits, and more (Lynch and McConnell 2002; Yildirim et al. 2006a, 2006b; Yildirim et al. 2010; dos Santos et al. 2011; Ahn et al. 2012; Veire et al. 2012). Hence, epigenetic events should be expected to modify the gene regulatory networks of DPSC niche(s). Moreover, it has been shown that MSC can also specifically interact with a large variety of immune cells and may mediate immune modulatory processes. Moreover tight controls of regulatory networks gradually deteriorate with aging (Lepperdinger 2011; Le Blanc and Mougiakakos 2012). The heterogeneous population of dental pulp reacts by several mechanisms to those factors that might influence stem cell quality within the niche. Therefore, deciduous teeth and young permanent teeth might be better sources for stem cell isolation.

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Chapter 5

Isolation Methods of Dental Pulp Stem Cells

Easy and quick isolation of the most primitive stem cell populations from available tissues is warranted for tissue engineering. The most reliable cell source for dental tissue engineering is that of autologous pulp stem/progenitor cells isolated from deciduous or primary teeth. The ease of isolation and high expansion potential in vitro has made them very attractive as a model system for many researchers.

While the lack of universally accepted markers continues to be the major obstacle to isolating stem cells, there is also no consistent isolation and/or purification method for DPSC. Controversies still remain in obtaining reproducible results by the published methods especially where the differentiation protocols are concerned (Iohara et al. 2008; Hirata et al. 2010; Suchanek et al. 2010; Yu et al. 2010). Meanwhile, different isolation methods have striking impacts on the differentiation potential of adult stem cells (Liu et al. 2006; Bakopoulou et al. 2011). The lack of consistency between the established protocols of different laboratories adds to the challenge of interpreting previously reported data.

5.1 Isolation of Dental Pulp Stem Cells

Extracted teeth by routine dental practice and exfoliated deciduous teeth by physiological resorption are dental pulp sources. Isolation of dental pulp stem is quite easy and completely noninvasive: The dental crown should be disinfected briefly before extraction with 0.3 % chlorhexidine and after extraction with iodine and 70 % ethanol. Teeth should be placed into a transport medium, if transported to another lab for isolation procedures. Transport medium should include at least 3 % (w/v) penicillin and streptomycin and 5 µg/mL amphotericin B. Since the oral cavity has the second highest proportion of microbiomes in humans (Group 2012), all instruments should be sterilized against the risk of a microbial contamination. Permanent teeth should be cracked with a mini hammer in order to get access to the

pulp chamber and root canals. The crowns of resorbed deciduous teeth may be cracked as well, since the pulp tissue remnants reside in the pulp chamber. Once a clear visual access is established, the pulp tissue can be taken out with toothless surgical pliers.

There are mainly two isolation methods for DPSC: enzyme digestion and the outgrowth method.

5.1.1 Enzyme Digestion Method

After washing with sterile phosphate buffered saline (PBS), pulp tissue should be cut into as small pieces as possible (~1 mm³ pieces). Tissue pieces should be washed twice with sterile PBS and placed in a solution of 2 mg/mL collagenase type I and dispase for 30–45 min at 37 °C with gentle agitation on an orbital shaker (Oktar et al. 2011). Cell suspensions can be obtained by passing the tissues through a 70-µm cell strainer. Alternatively, the homogenate should be centrifuged at 130×g for 5 min, and the pellet should be resuspended in the growth medium. Then, T25 cm² cell culture plates can be used to culture and expand DPSC up to several passages.

There are different enzymatic combinations such as 3 mg/mL collagenase type I and 4 mg/mL dispase for 30–60 min at 37 °C (Liu et al. 2006; Huang et al. 2006; Tirino et al. 2011), 1 mg/mL collagenase type I and 2.4 mg/mL dispase (Lee et al. 2011) 2mg/mL collagenase type I and dispase for 30 min at 37°C (Yildirim et al. 2012) or 0.2 % trypsin for 5 min at 37 °C (Spath et al. 2010) or 3 % collagenase type I for 1 h 37 °C (Yan et al. 2011).

5.1.2 Outgrowth Method

In this method, pulp tissue is cut into pieces and placed directly in a cell culture plate with the growth medium.

Although both methods appear to give rise to pulp cells that have stem cell properties (About et al. 2000; Couble et al. 2000; Gronthos et al. 2000; Batouli et al. 2003), the effects of various isolation methods on pulp cell populations have also been questioned. One of the earliest studies by Nakashima showed that four different enzymatic separation (trypsin, collagenase with and without trypsin treatment, and a trypsin–collagenase mixture) had no effect on the morphological characteristics of the cultured pulp cells, but the trypsin pretreatment/collagenase method gave the highest number of isolated cells (Nakashima 1991). Nonetheless, the enzymatic digestion method allows different cell types to develop. While Nakashima reported three different morphologies as spindle, stellate, and epithelial-like cells (Nakashima 1991), Huang et al. showed compact and loose colony-types after enzymatic digestion (Huang et al. 2006). Since enzymatic digestion causes all cell types to be released from the tissue, it is not surprising to observe

different cells. However, only cells that have a fibroblastic appearance were able to survive after several passages because of the usage of mesenchymal cell promoting culture media (Huang et al. 2006). On the other hand, Bakopoulou et al. showed that in the enzymatically digested samples, the mineralization rate and the amount of mineralized matrix produced were higher compared to outgrowth cultures. However, the cell culture media was different for the digested and outgrown cultures (Bakopoulou et al. 2011). Contrarily, Spath et al. displayed enhanced differentiation abilities with explant cultures when compared to enzymatically digested DPSC (Spath et al. 2010). However, they cultured trypsin pretreated explants onto a Petri dish coated with fibronectin in MegaCell complete medium. Furthermore, Kerkis and Kaplan reported that they obtained immature dental pulp stem cells (IDPSC) from deciduous teeth by using enzymatic digestion and DMEM low glucose supplemented with 10 % FBS, while the outgrowth method using DMEM/F12 supplemented with 15–20 % FBS gave IDPSC (Kerkis and Caplan 2012). While it is rational to think that SHED is heterogeneous compared to single cell-derived colonies of IDPC, wider screening gene expressions of the latter do not prove that these two isolation systems gave different cells (Kerkis and Caplan 2012). Moreover, Kerkis et al. only screened the deciduous dental pulp cells isolated via the outgrowth method and compared their data (Kerkis et al. 2006) with the ones obtained by the other groups, which does not include the same set of genes (Miura et al. 2003; Pivoriunas et al. 2010; Yamaza et al. 2010). As a matter of fact, Bakopoulou et al. reported more favorable results for stem cell properties of enzymatic digestion methods of SHED over outgrowth method (Bakopoulou et al. 2011). In conclusion, there is an agreement that almost all DPSC populations show complete or partial overlap in the expression pattern of analyzed markers (Huang et al. 2009; Kerkis and Caplan 2012).

The most significant differences for enzymatic digestion and outgrowth methods are in reported cell number and homogeneity. While the former is always higher in enzymatic digestion, tissue explants allow the expansion of fibroblastic-like cells in dental pulp cultures.

Since there are no identified dental pulp stem cell marker(s), there are continuing efforts for the further purification of isolated dental pulp cells. Yan et al. modified the enzyme digestion method by utilizing a size-sieved protocol in order to get small-sized cell populations containing a high percent of stem cells (Yan et al. 2011). The alternatively developed colony culture method is not only time consuming, but also obtained colonies are not uniform (Gronthos et al. 2000).

The other and commonly used techniques are magnetic and fluorescent activated cell sorting (FACS). While magnetic activated cell sorting seems to be simple and inexpensive, FACS offers high purity and viability. However, electrical charges applied to the cell, resultant semi-sterility, and high cost are the drawbacks of the FACS method (Tirino et al. 2011).

Since the STRO-1 antibody identifies a cell surface antigen expressed by the osteogenic fraction of stromal precursors in human bone marrow, it has been used to select the stromal cell precursor of pericyte cells within dental pulp (Yang et al. 2007). While the STRO-1⁺ fraction can give cells with both odontogenic and

multilineage potential in rat dental pulp (Yang et al. 2007), Shi and Gronthos have shown that STRO-1⁺ DPSC express vascular antigen CD146, α -smooth muscle actins, and a pericyte-associated antigen (3G5), by immunohistochemistry, FACS, and/or immunomagnetic bead selection (Shi and Gronthos 2003).

Laino et al. showed that c-kit⁺/CD34⁺/CD45⁻ DPSC in vitro were capable of woven bone tissue formation (Laino et al. 2005). Even supposing CD34 is a hematopoietic progeny detector, Laino et al. reported that instead of STRO-1, which detects only a 5 % fraction of CD34⁺ stem cells (Simmons and Torok-Storb 1991), they used CD34⁺ cells not only because it is a marker for primitive pluripotent stem cells stromal precursors, but also CD34 positivity corresponds to only up to 3 % of the population (Zhan et al. 2004).

Furthermore, Hoechst 3342-sorted SP cells from porcine dental pulp have been shown to possess multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis (Iohara et al. 2006). The same group compared the CD31⁻ CD146⁻ and CD31⁺ CD146⁻ SP cells, and found that the former sub-fractions showed stronger neurogenic potential, while the adipogenic, chondrogenic, and odontogenic differentiation potential was similar in the two sub-fractions of SP cells. Moreover, they have shown the sub-fraction demonstrating CD34⁺ and vascular endothelial growth factor-2 (VEGFR2)/Flk1⁺ promoted vasculogenesis in models of mouse hind limb ischemia (Iohara et al. 2008).

5.2 Cell Culture Media for Dental Pulp Stem Cells

Medium can influence the manipulation and culture techniques as well as morphology and differentiation ability of the cells especially for the culture of heterogeneous cell populations that contain cells at various stages of differentiation as in dental pulp. Dental pulp cells are usually cultured in Eagle's basal medium (EBM), Eagle's minimum essential medium (MEM), α -MEM, or Dulbecco's modified Eagle medium (DMEM). A more complex Ham's F12 nutrient medium (F12) was optimized for cloning. A 1:1 mixture of DMEM and F12 combine the richness of F12 and the higher nutrient concentration of DMEM. These support the expansion of the mesenchymal cells without inducing early senescence and differentiation (Freshney 2005).

It has been shown that various media favor different cell populations in cultures of pulpal cells. While Medium 199 favors fibroblastic cells, MEM and BME support many connective tissue and epithelial cells (Miller et al. 1976). Nakashima found that the DMEM and the DMEM/F-12 mixture led to the best results in adhesion, cell growth, and cell number at confluence when compared with F-12, Roswell Park Memorial Institute medium (RPMI) and Medium 199 (Nakashima 1991). Initially Nakashima concluded that DMEM may be preferred for culturing pulp cells, because the resulting population ratio and labeling index showed that it supported a population of the prominently fibroblastic pulp cells. However, later her group used very complex media for porcine deciduous pulp cultures (Iohara et al. 2008). Even though their

enzymatic digestion was not described clearly, the media was EBM2, including growth factors such as basic bFGF, insulin-like growth factor 1 (IGF1), epidermal growth factor (EGF), and VEGF-A. In addition, the optimal concentration of porcine serum was determined to maintain all the sorted cells (Iohara et al. 2008). Although they used DMEM in their consequent studies, the serum ratio was 2 % (Iohara et al. 2008) or 10 % (Ishizaka et al. 2012) and supplements were not indicated.

Lopez-Cazaux et al. showed that while calcium-rich and phosphate-poor medium MEM supported recruitment and/or proliferation of smooth muscle actin positive (SMA⁺) cells, high-calcium medium RPMI 1640 lowered the SMA⁺ cells in human dental pulp cultures and the authors reported that MEM was more potent for odontoblast-like cell differentiation than RPMI 1640 (Lopez-Cazaux et al. 2006). Govindasamy et al. cultured dental pulp cells in four different media: DMEM-knock out (DMEM-KO), DMEM low glucose (DMEM-LG), DMEM/F12, and minimum essential medium- α (α -MEM), all supplemented with 10 % FBS. They observed that α -MEM and DMEM-KO are the most optimal culture conditions for DPSC in terms of their proliferation, morphology, cell surface marker analysis, and population doubling time. The interesting result from this study is that while cells grown in α -MEM, DMEM-KO, and DMEM/F12 showed differentiation into mesoderm lineages at early passage (P1) and late passage (P9), DPSC cultured under DMEM-LG has been reported to exhibit differentiation defects when the cells were differentiated after passage 5 (Govindasamy et al. 2010).

In our laboratory we use the outgrowth method with DMEM/F12 plus 10% FBS for both SHED and DPSC: starting from their first days in culture, DPSC show the spindle shaped, typical fibroblast-like appearance that is consistent through several passages (over 35) (Fig. 5.1).

Kerkis and Kaplan stressed that:

In cell populations isolated and cultured in vitro, the balance in quantity of stem cell and progenitors at the different stages of commitment directly depends on the method of isolation and cell culture conditions. Accordingly, quality and quantity of fetal bovine serum (FBS) added in basal culture medium, use of enzymatic digestion, addition of growth factors, and other changes introduced into the protocol for stem cell isolation can contribute to selection or propagation of different populations of stem cells and/or progenitors (Kerkis and Caplan 2012).

Likewise, amino acid, protein, glucose, and vitamin contents of media might be responsible for different outcomes. Our preliminary data about the effects of albumin on DPSC proliferation showed that albumin has a strong influence on cell morphology (Fig. 5.2).

In a nutshell, the techniques used to obtain DPSC differ widely, even within the same group. These variations make comparison difficult: neither the same techniques nor cell culture media being used consistently (Table 5.1). Although very widely screened data are available within the literature with regard to the cellular and molecular characteristics of expanded cell cultures, different techniques definitely cause different and more confusing results. Therefore the interpretation of the data needs careful evaluation. Researchers might focus on their needs in the published data, not only on already interpreted results.



Fig. 5.1 Typical fibroblastic-like appearance of DPSC (inverted phase-contrast microscopy, 400 $\times$)

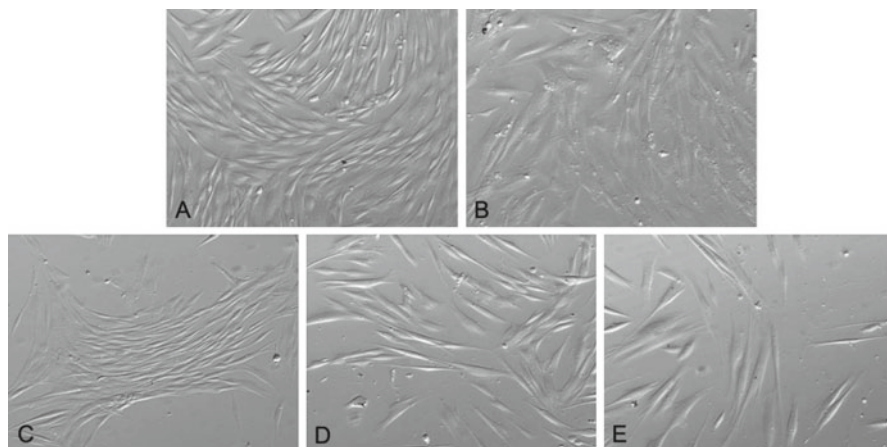


Fig. 5.2 The effects of albumin on cell behavior. (a) Serum-free media including 0.05 % albumin, (b) DMEM/F12, (c, d, and e) serum-free media + 1 %, 0.5 %, and 0.1 % human serum albumin, respectively. Albumin causes bridge like connections between cells without influencing elongated fibroblastic appearance and proliferation (inverted phase-contrast microscopy, 200 $\times$, Yildirim et al. unpublished observation)

Table 5.1 Different techniques for DPSC and SHED isolation and growth

Group	Isolation	Growth medium	Selection	Differentiation into	Plus
DPSC (Gronthos et al. 2000)	3 mg/mL collage-nase type I/4 mg/mL dispase	α-MEM + 20 % FCS, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, P/S		Osteogenic cells	Characterization of the immunophenotype and generation a dentin-like structure lined with human odontoblast-like cells that surrounded a pulp-like interstitial tissue in immunocompromised mice
DPSC (Liu et al. 2006)	3 mg/mL collage-nase type I/4 mg/mL dispase	α-MEM + 10%FCS, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, P/S	Magnetic STRO-1 ⁺ , 3G5 ⁺ , CC9 ⁺	Osteogenic, adipogenic, neurogenic cells	
DPSC (Honda et al. 2007)	Tissue explants	α-MEM + 10 % FBS, 1× Glutamax, 50 μg/mL L-ascorbic acid phosphate magnesium salt <i>n</i> -hydrate, P/S	FACS Hoechst 33342	Osteogenic cells	Expression of: ABCG2, Nestin, Notch-1, α-SMA
DPSC (Spath et al. 2010)	Trypsin pretreated tissue explants	MegaCell DMEM complete medium + 10 % FCS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, P/S		Osteogenic, chondrogenic, myogenic cells	Contribution of human DPSC to regenerating muscle in mice
DPSC (Ishizaka et al. 2012)	Specific enzyme is not identified	DMEM + 10 %FBS	FACS CD31 ⁺	Angiogenic, neurogenic cells	Regeneration of the pulp tissue in pulpectomized root canals in dogs

(continued)

Table 5.1 (continued)

Group	Isolation	Growth medium	Selection	Differentiation into	Plus
SHED (Miura et al. 2003)	3 mg/mL collage-nase type I/4 mg/mL dispace	α -MEM + 20 % FCS, 100 μ M L-ascorbic acid 2-phosphate, 2 mM L-glutamine, P/S		Neural, adipogenic, odontogenic cells	After in vivo transplantation, induction of bone formation, generation of dentin, and survival in mouse brain along with the expression of neural markers
SHED (Kerkis et al. 2006)	3 mg/mL collage-nase type I/4 mg/mL dispace	(DMEM)/Ham's F12 (1:1) + 15 % FBS, 2 mM L-glutamine, and 2 mM nonessen-tial amino acids, P/S		Neuronal, chondrogenic, osteogenic, myogenic cells	Expressions of embryonic stem cell markers Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81
SHED (Bakopoulou et al. 2011)	3 mg/mL collage-nase type I/4 mg/mL dispace	α -MEM + 15%FBS, 100 μ M L-ascorbic acid, 2 mM L-glu-tamine, P/S, 0.25 mg/mL amphotericin B		Odontogenic, osteogenic cells	
SHED (Bakopoulou et al. 2011)	Tissue explant	DMEM + 10 % FBS, P/S, 0.25 mg/mL		Odontogenic, osteogenic cells	
SHED (Wang et al. 2012)	0.3 mg/mL collagenase type I/0.1 % dispace II	DMEM + 15 % FBS, P/S		Osteogenic, adipogenic cells	Enhanced potential to form bone after transplantation with ceramic bovine bone into subcutaneous of immunocompromised mice

DPSC dental pulp stem cell, *SHED* stem cells from human exfoliated deciduous teeth, α -MEM alpha modification of Eagle's medium, *DMEM* Dulbecco's modified Eagle's medium, *FBS* fetal bovine serum, *FCS* fetal calf serum, *P/S* 100 U/mL penicillin, 100 μ g/mL streptomycin

5.3 Cryopreservation of DPSC

Cryopreservation is a process where cells or whole tissues are preserved by cooling to very low subzero temperatures (typically -196°C). This allows cells to restart proliferation, differentiation, and new tissue formation for therapeutic use. At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. However, when cryoprotectant solutions are not used, the cells being preserved are often damaged during freezing or thawing. Conventional, multistep slow freezing, and vitrification methods were comparatively tested and an efficient and reliable cryopreservation method for DPSC resulting in the high cell survival rate has been reported (Papaccio et al. 2006; Perry et al. 2008; Woods et al. 2009; Lee et al. 2010a, 2012a, b; Zhurova et al. 2010; Chen et al. 2011). Reports show that dental pulp tissues of cryopreserved teeth are not able to maintain their biological properties because of the limitation of permeability of cryopreservative agent into pulp cavity (Osathanon 2010). However, it has also been shown that the isolation rate of dental pulp cells from cryopreserved teeth is quite high (Lee et al. 2010a; Gioventu et al. 2012). Although cryopreservation methods are currently sufficient for cell research purposes, in my opinion more concrete and successful clinical evidence of their indispensable benefits for whole tooth and isolated DPSC banking facilities for future regenerative purposes is required.

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Chapter 6

Characterization of DPSC

6.1 Phenotypic Characteristics

Both SHED and DPSC have indistinguishable fibroblastic characteristics (Fig 6.1). They preserve plasticity during at least 25 passages, while maintaining the normal karyotype and the rate of expansion characteristic of stem cells (Kerkis et al. 2006).

Using flow cytometry, cell surface markers analysis of DPSC shows consistently that they are positive for CD73, CD90, CD105 (endoglin), CD13, CD29 (integrin b1), CD44, HLA-A, B, and C. They are negative for CD45, CD34, CD14, CD54, CD 133 (Oktar et al. 2011; Chen et al. 2012), thus they fulfill the criteria of MSC set by the ISCT (Dominici et al. 2006). Besides confirming the findings described above, it has been shown that SHED and DPSC express pluripotency markers including Oct4, Nanog, c-Myc, Sox2, stage specific embryonic antigens (SSEA-3, SSEA-4), and tumor recognition antigens (TRA-1–60 and TRA-1–81) (Kerkis et al. 2006; Nakamura et al. 2009; Govindasamy et al. 2010; Liu et al. 2011). However, it is important to note that these expression levels might not be compatible with the values observed in embryonic stem cells (Lengner et al. 2007). For further in vitro phenotypic characteristics of DPSC, the paper from Huang, Gronthos, and Shi provides clear ideas regarding the differences between DPSC, SHED, and the other tooth-originated stem cells (Huang et al. 2009).

Moreover, a cDNA microarray system-based comparison between human DPSC and human MSC has been published recently (Yamada et al. 2006). Broadening the previous gene expression profile study for 4,000 human genes in DPSC and bone marrow stroma stem cells by Shi et al. (2001), Yamada et al. revealed several genes which encode the extracellular matrix components, cell adhesion molecules, growth factors, and transcription factors including Msx1 and Jun-B. According to their cluster analyses Yamada et al. concluded that there are differences in gene expression levels for cell signaling, cell communication, and cell metabolism (Yamada et al. 2006). Using integrated miRNA and mRNA expression profiles, Chen et al. compared DPSC and UCSC (Chen et al. 2012). They determined by functional network analyses that two

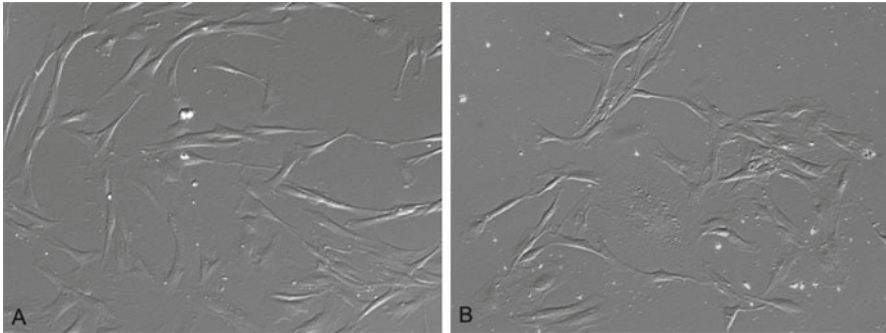


Fig. 6.1 Indistinguishable fibroblastic phenotypes of SHED (a) and DPSC during the first days in culture (b) (inverted phase-contrast microscopy, 400 $\times$)

genes (SATB2 and TNFRSF11B), which are involved in ossification, bone development, and actin cytoskeleton organization, are signatures of DPSC. They also showed upregulations of NEDD4 and EMP1, which are shown to be involved in neuroectodermal differentiation in DPSC (Chen et al. 2012). Additionally, Pivoriunas et al. revealed cytosolic and nuclear proteins of SHED and reported a close resemblance between SHED and MSC-like cells from other tissues (Pivoriunas et al. 2010). Despite the fact that dental pulp is a heterogeneous tissue, availability of “omics” data might help to develop more targeted isolation methods of DPSC in the future.

6.2 Multidifferentiation Capacity

DPSC are multipotent since they are capable of giving rise to various lineages of cells, when exposed to the appropriate environmental cues. Most of the DPSC have been shown to differentiate *in vitro* into the desirable cells through a well-defined transcriptional cascade. Albeit the majority of the studies have focused on the ability of DPSC to differentiate into odontoblast/osteoblast-like cells (Couble et al. 2000; Gronthos et al. 2000; Laino et al. 2005; Cordeiro et al. 2008; Bakopoulou et al. 2011; d’Aquino et al. 2011; Min et al. 2011), they can differentiate into functionally active neurons (Arthur et al. 2008; Kadar et al. 2009; Kiraly et al. 2011; Nourbakhsh et al. 2011; Osathanon et al. 2011), mature melanocytes (Stevens et al. 2008; Paino et al. 2010), smooth muscle cells (Kerkis et al. 2006; d’Aquino et al. 2007; Gandia et al. 2008), islet-like aggregates (Govindasamy et al. 2011), and hepatic cells (Ishkitiev et al. 2010) as well.

The classical differentiation recipes are as follows: Osteogenesis can be initiated experimentally with DMEM-LG (low glucose) with 10 % (v/v) FBS, 100 nM dexamethasone, 0.2 nM ascorbic acid, 10 mM β -glycerophosphate; in the case of chondrogenesis 10 ng/mL TGF β 3, 100 nM dexamethasone, 50 μ g/mL ascorbic acid, 1 mM sodium pyruvate, 6.25 μ g/mL insulin, 6.25 μ g/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin, and 5.35 mg/mL linoleic acid in

DMEM-high glucose can be used. Last but not least, adipogenesis can be initiated with the xenobiotics such as 1 μ M dexamethasone, 500 μ M IBMX, 60 μ M indomethacin, and 5 μ g/mL insulin in DMEM-LG supplemented with 10 % FBS. Oil red O staining of 4 % PFA fixed cells and toluidin blue staining in cryosections of cell nodules in micropellet systems are universally accepted visualization methods for lipid droplets in adipogenically induced cells, and accumulation of extracellular matrix composed of mucopolysaccharides in chondrogenically-induced cells, respectively (Karahuseyinoglu et al. 2007a, 2008; Oktar et al. 2011; Yildirim et al. 2012). Despite some pitfalls in von Kossa and Alizarin Red S (Bonewald et al. 2003; Wang et al. 2006), they are still in use for expected collagenous extracellular matrix formation and mineralization in osteogenically induced cells (Karahuseyinoglu et al. 2007a, 2008; Oktar et al. 2011). While von Kossa shows only calcium accumulations, Alizarin Red S reacts with calcium cations to form a chelate. Although other stains such as two fluorescent dye combinations (xylene orange and calcein blue) have been suggested, staining methods alone are not appropriate for the identification and quantification of bonelike minerals. Hence, using more specific diagnostic techniques such as X-ray diffraction, electron microscopy, or Fourier transform infrared spectroscopy (FTIR) may better verify the presence and quality of calcium phosphate phases (Bonewald et al. 2003).

6.2.1 Problems in Adipogenesis

While most of the differentiation assessments revealed strong osteogenic and chondrogenic potential for DPSC (Gronthos et al. 2000, 2002; Huang et al. 2006; Iohara et al. 2006; Kerkis et al. 2006; Liu et al. 2006; Honda et al. 2007), there are no consistent reports of their adipogenic potential (Gronthos et al. 2000; Pivoriunas et al. 2010; Oktar et al. 2011; Struys et al. 2011). We compared the differentiation potentials of human DPSC and UCSC (Oktar et al. 2011). While chondrogenic and osteogenic differentiation of both cell types were similar, their adipogenic differentiation within the same conditioned media resulted in different outcomes. While UCSC started to display specific intracytoplasmic lipid granules in the central cytoplasm after only 48–72 h following adipogenic treatment, DPSC protected their fusiform shape without showing any lipid granules even after prolonged treatments of up to 5 weeks. Furthermore, following successful adipogenic induction of UCSC, we found that nucleostemin (NS), which has been shown as a nucleolar protein expressed in stem and cancer cells, could no longer be detected in forming preadipocytes. However DPSC that did not respond to adipogenic induction displayed a stable NS expression (Oktar et al. 2011). Other groups also noted the non-responsiveness of DPSC to adipogenic stimuli. Pivoriunas et al. reported the same results for adipogenic differentiation of DPSC after several trials (Pivoriunas et al. 2010).

Although DPSC have been reported to possess an adipogenic differentiation capacity under our conditions (Huang et al. 2009; Koyama et al. 2009), this discrepancy may be due to several factors including individual differences. The environmental

conditioning or predisposition of the stem cells might be reflected in their distinctive differentiation ability. Their fate *in vivo* might be dependent of their embryonic origin and their anatomical localization. Moreover, teeth are derived from the ectoderm of the first branchial arch and the ectomesenchyme of the neural crest. Deciduous teeth start to form between the sixth and eighth weeks of gestation, and permanent teeth begin to form in the twentieth week (Thesleff et al. 1995). Umbilical cord, on the other hand, develops from the extra-embryonic mesoderm at days 12–13 after fertilization. Likewise, Struys et al. claimed that dental pulp is confined by a hard dental tissue (dentin) which is more likely to create a tendency for osteogenic events in those cells (Struys et al. 2011). However, the observed strong osteogenic response of umbilical cord intervacular cells in our experimental conditions does not support this hypothesis (Yildirim et al. 2012). Osteogenic differentiation was robust in both MSC, while the staining pattern of von Kossa was completely different. DPSC displayed more heterogeneous widespread small, round, and dark stained mineralized structures, whereas UCSC showed bone-nodule like formation (Yildirim et al. 2012). Therefore, the diverse developmental lineage and differential potential of UCSC and DPSC may reflect stem cell heterogeneity. It has been suggested that the propensity of mesenchymal stem cells derived from various tissue sources toward a specific lineage is different from each other (Kerkis and Caplan 2012). This hypothesis deserves further exploration.

6.2.2 Odontogenic Differentiation

Spontaneous differentiation of DPSC toward several lineages has been shown (Kerkis et al. 2006; Paino et al. 2010; Yu et al. 2010; Lizier et al. 2012). Odontoblasts are not one of those lineages assuming that the heterogeneous nature of DPSC cultures, and growth and proliferation conditions could not provide the necessary permissive signals for odontoblast terminal differentiation. Papaccio's group has shown that in the presence of 20 % of FBS, bone is the main commitment of dental pulp stem cells, but not dentin (Laino et al. 2005, 2006; Papaccio et al. 2006; d'Aquino et al. 2007). On the other hand, DPSC and SHED have been shown to have odontogenic potential with several different *in vitro* and *in vivo* evaluation methods, in addition to quite different isolation, culturing, and differentiation methods (Table 6.1). What is more, no specific marker(s) is known to differentiate between odontoblasts and osteoblasts. While beta-glycerophosphate, ascorbic acid, and dexamethasone have been used for odontoblast differentiation, researchers are trying to find a unique way for *in vitro* odontoblast differentiation. Table 6.1 shows different odontogenic induction techniques and evaluation methods.

Many other factors have been shown to promote odontoblastic differentiation of DPSC, such as porcine tooth germ conditioning medium (Wang et al. 2011), the combination of MTA and enamel matrix derivatives (Min et al. 2009), FG2, TGF β 1,

Table 6.1 Evaluation of odontogenic differentiation by different research groups

Group	Odontogenic induction by	Evaluation markers	Evaluation methods	Results
Couple et al. (2000)	Culturing DPSC in EBM supplemented with 10 % or 15 % FCS ± β-GP	Coll type I, DSP	Electron microscopy, X-ray microanalysis, RT-PCR	Inducing odontoblastic features including spatial organization similar to odontoblastic layer
Gronthos et al. (2002)	In vivo: transplantation of DPSC with HA/TCP ceramic powder subcutaneously into immunocompromised mice		Histology	Generation of ectopic dentin/pulp-like complexes
Batouli et al. (2003)	Transplantation of DPSC with HA/TCP ceramic powder subcutaneously into immunocompromised mice	DSP	Histology, histochemistry	Formation of vascularized pulp-like tissue surrounded by a layer of odontoblast-like cells expressing DSP
Huang et al. (2006)	DPSC cultured in α-DMEM including 20 % FBS 2 mM KH ₂ PO ₄ , 20 mM HEPES, and 100 nM dexamethasone; or these three chemicals plus 5 × 10 ⁻⁸ M Vit D3		Alizarin red S staining	Promoted mineral nodule formation
Cordeiro et al. (2008)	SHED seeded in dentin slice/ PLLA scaffolds were transplanted into immunodeficient mice	Human factor VIII, DSP	Histochemistry, B-galactosidase staining, TEM	Pulp-like tissue with functional blood vessels lined with odontoblastic cells
Nam et al. (2011)	DPSC were populated on porous calcium phosphate granules	DSP, Col type I, DMP1, OCN	RT-PCR, WB, Alizerin red S staining	Odontogenic development supported by the upregulation of DSP, DMP-I, Coll type I, and OCN

(continued)

Table 6.1 (continued)

Group	Odontogenic induction by	Evaluation markers	Evaluation methods	Results
Lee et al. (2011)	Preameloblast-derived factors	DSPP, BSP, Nestin, Coll type I, RUNX2, ALP, OC, mCpne7	Real time PCR, WB, Alizerin red S staining, dentin sialophosphoprotein promoter activity, liquid chromatography–mass spectrometry In vivo: transplantation into immunocompromised mice	Facilitated odontoblast differentiation and generation of pulp-like structures lined with human odontoblast-like cells showing typical odontoblast processes

BME Eagle's basal medium, *Coll type I* collagen type I, *OCN* osteocalcin, *DSP* dentin sialoprotein, *DSPP* dentin sialophosphoprotein, *DMP-1* dentin matrix protein-1, β -*GP* beta-glycerophosphate, *TEM* transmission electron-microscopy

BMP-2, GDF-11, DMP-1 derived peptide, asporin and EphB/ephrin-B (Iohara et al. 2004; Nakashima et al. 2004; Yang et al. 2007; He et al. 2008; Arthur et al. 2009; Chaussain et al. 2009; Yang et al. 2009; Casagrande et al. 2010), inhibition of Delta 1 (Wang et al. 2011), TWIST1 over expression (Li et al. 2011), Bmi-1 transduction (Mehrazarin et al. 2011), the inhibition of mammalian target of rapamycin (mTor) complex Torc 2 (Kim et al. 2011), pro-inflammatory cytokines (IL-1 and TNF) (Yang et al. 2012), simvastatin (Okamoto et al. 2009), conditioned medium extracted from tooth germ cells (Duailibi et al. 2004; Ohazama et al. 2004; Yu et al. 2006a, b), natural mineralized scaffolds (Wang et al. 2010; Zhang et al. 2012), and even oral bacterial extracts (Abe et al. 2010).

Since mechanical forces have effects on the differentiation of MSC (Bayati et al. 2011; Sarraf et al. 2011), there are also reports that evaluate behavioral differences under mechanical stress. While Yu et al. showed that odontoblastic differentiation potential of DPSC is enhanced under dynamic hydrostatic pressure (Yu et al. 2009), Cai et al. reported that unaxial cyclic tensile stretch inhibits osteogenic and odontogenic differentiation of DPSC (Cai et al. 2011). The effect of oxygen tension on proliferation and differentiation of DPSC has been surrogated and shown that hypoxic conditions (3 % O₂, 5 % CO₂, and 92 % N₂) increased the proliferation, but inhibited the differentiation capacity to odontoblasts (Iida et al. 2010).

Although the mechanism of odontoblastic differentiation is not known, many researchers are doing further investigations in order to discover any odontoblast-specific gene expressions of those morphologically odontoblast-like cells.

Examining tooth development in Runx2 transgenic mice, Miyazaki et al. showed that Runx2 not only inhibits the terminal differentiation of odontoblasts, but also induces trans-differentiation of odontoblasts into osteoblast (Miyazaki et al. 2008). Runx2, also known as core-binding factor subunit alpha-1, is a key transcription factor associated with osteoblast differentiation (Komori et al. 1997). Although it is essential in tooth development and abundantly expressed in dental pulp tissue (Yildirim et al. 2008; Chen et al. 2009), its function in odontoblast differentiation is unclear. The role of Runx2 in the regulation of odontoblast differentiation is hypothesized by Komori (2010) (Fig 6.2).

Moreover, it has been shown that TWIST-1 and TWIST-2 suppress the activity of Runx2 and thereby regulate bone formation (Bialek et al. 2004; Kronenberg 2004). Likewise Li et al. showed that overexpressing or silencing TWIST1 levels could tune the differentiation capacity of DPSC effectively (Li et al. 2011). In this sense, TWIST1 and RUNX2 are seen to be unique candidates for probing odontoblast lineage commitment, as Sui Huang group's pioneering studies confirmed that lineage specification of multipotent progenitor cells is governed by a balance of lineage-affiliated transcription factors (Huang et al. 2007; Zhou et al. 2011). In developmental or repair events, the progenitor cell(s) of dental pulp can be directed in the choice between osteoblast and odontoblast fates via RUNX2 or TWIST-1 couples.

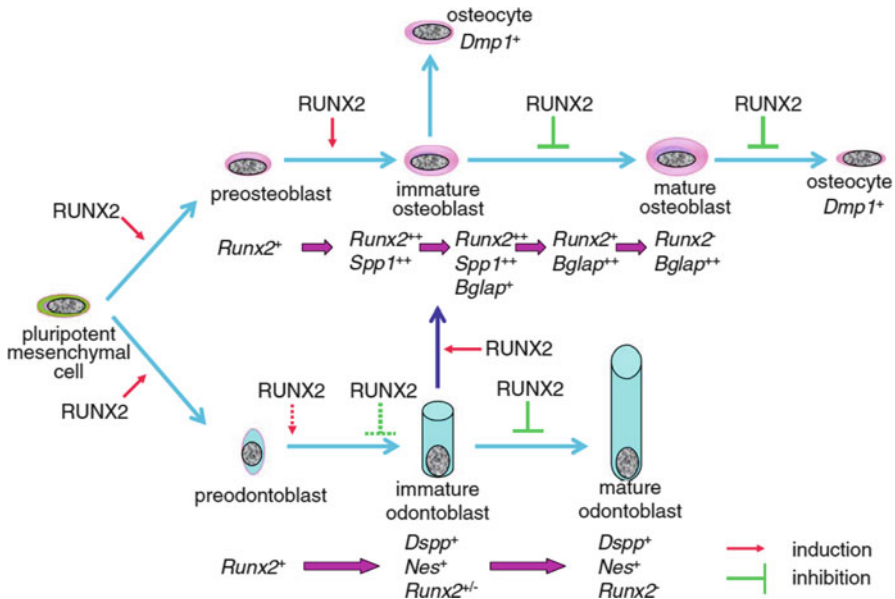


Fig. 6.2 Regulation of osteoblast and odontoblast differentiation by RUNX2. RUNX2 directs pluripotent mesenchymal cells to the osteoblast lineage, increases the number of immature osteoblasts, but inhibits osteoblast maturation. Preosteoblasts express Runx2. Immature osteoblasts express Runx2 and Spp1 and, subsequently, Bglap. Mature osteoblasts express Bglap, but Runx2 expression is down-regulated. Osteocytes express Dmp1. The transition of immature osteoblasts to osteocytes occurs at an early stage of bone development. The common precursors of osteoblasts and odontoblasts are restricted to neural-crest-derived mesenchymal cells, but the basal process of osteoblast differentiation is similar in the neural-crest-derived and non-neural-crest-derived pluripotent mesenchymal cells. Preodontoblasts differentiate from neural-crest-derived pluripotent mesenchymal cells. RUNX2 is essential for differentiation of pluripotent mesenchymal cells into preodontoblasts. RUNX2 also probably induces the differentiation of preodontoblasts into immature odontoblasts at an early stage but is inhibitory at a late stage. Preodontoblasts express Runx2, immature odontoblasts express Dspp and Nes but Runx2 weakly, and mature odontoblasts express Dspp and Nes but not Runx2. Runx2 expression is down-regulated during odontoblast differentiation, and RUNX2 inhibits terminal differentiation of odontoblasts. Overexpression of Runx2 induced transdifferentiation of odontoblasts to osteoblasts (*Spp1* secreted phosphoprotein 1/osteopontin, *Bglap* bone gamma carboxyglutamate [gla] protein/osteocalcin, *Dspp* dentin sialophosphoprotein, *Nes* nestin, *Dmp1* dentin matrix protein 1). Reproduced from Komori (2010) with permission of the publisher

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Chapter 7

Reprogramming of DPSC to Induced Pluripotent Stem Cells

Generating a pluripotent cell in vitro by rewinding the internal clock of any somatic cell to an embryonic state and then forwarding its conversion into the desired differentiated cell fate represents a rational and ongoing approach in regenerative medicine (Yildirim 2012). There are available human tissues with no ethical or surgical concern, such as fat, blood, biopsy specimens, skin, plugged hair, and extracted teeth (Aasen et al. 2008; Sun et al. 2009; Ye et al. 2009; Yan et al. 2010).

DPSC could be effectively reprogrammed into induced pluripotent stem cells (iPSC; Yan et al. 2010; Beltrao-Braga et al. 2011) possibly because they are of mesectodermal origin expressing reprogramming factors inherently (Liu et al. 2011; Yildirim 2012). Our preliminary results clearly indicate that DPSC, SHED, and periodontal ligament stem cells (PDLSC) showing high single-cell clonogenicity responded well to the polycistronic vector, EF α -STEMCCA that includes Oct4, Klf4, Sox2, and cMyc. Using only morphological criteria (Blelloch et al. 2007; Maherali et al. 2007; Meissner et al. 2007) we can say that dental pulp and periodontal ligament-derived iPSC are like embryonic stem cells (ESC): small cells in the colonies with clear borders that separate them from the feeder layer (Fig 7.1). The expression of pluripotency markers of DPSC and SHED has been detected by RT-PCR (Fig 7.2, top). Albeit Sox-2 was not detected before reprogramming, it was consistent through passages in reprogrammed dental cells (Fig 7.2, bottom).

Rapid cycling parental dental pulp and PDL became smaller and continued to grow as monolayer on irradiated human foreskin fibroblast (HFF) feeder layer cells. Although it has not been detected immunocytochemically if most of the cells were expressing the reprogramming factors, the colony forming efficiency was high as they can be induced into the first morphological change of proper reprogramming events (Plath and Lowry 2011). While they were positive for alkaline phosphatase as an intermediate stage marker, continuous expression of the reprogramming factors through early passages may indicate that these cells are good candidates to overcome roadblocks toward pluripotency and to the maintenance of the iPS state, independent of factor expression (Brambrink et al. 2008; Papp and Plath 2011).

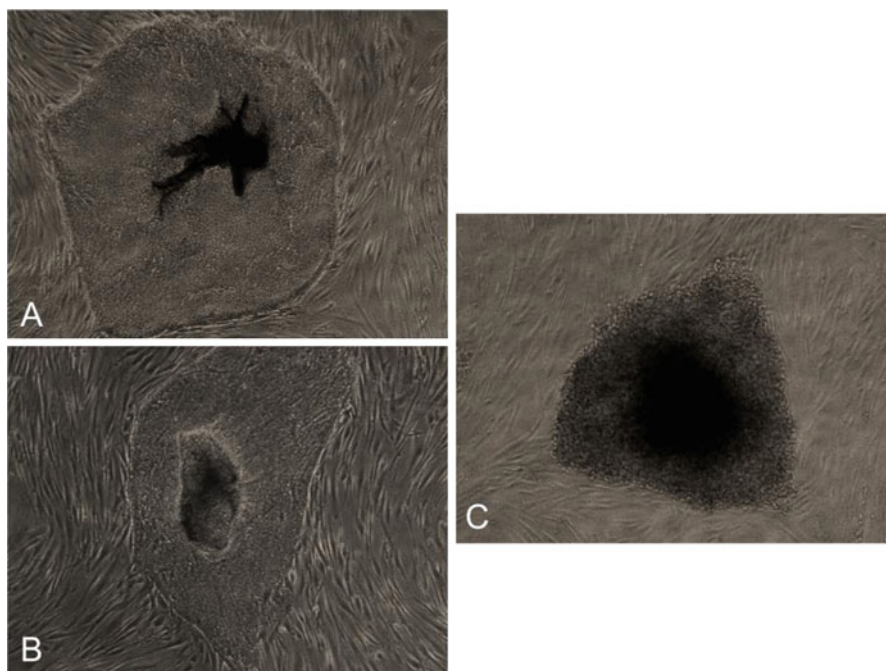


Fig. 7.1 iPSC colonies derived from (a) PDL stem cells, (b) SHED, and (c) DPSC (inverted 100 $\times$)

It is interesting to note that while SHED- and PDL-derived iPSC colonies have very similar morphologies, iPSC obtained from DPSC display compact dome-shaped morphologies as in mouse ESC. DPSC-derived ones have a different size and shape (Fig 7.2). Since there is no clear information about the differences between permanent and deciduous dental pulp tissues, it is hard to explain why colonies obtained from DPSC and SHED are so different. Yet, it has been proposed that MSC properties of PDL and DPSC are almost identical (Huang et al. 2009), which is in agreement with the morphological similarities of the SHED- and PDL-derived iPSC colonies in this study. The question as to whether those morphological discrepancies reflect epigenetic status of different but closely related cellular origins, from which they arose, deserves more attention.

In the process of reprogramming, as well as the regulation of checkpoints, the genome-wide alteration of epigenetic marks and mesenchymal–epithelial transition (MET) are required for the acquisition of pluripotency (Plath and Lowry 2011). Genetic factors, signaling pathways, and p53 pathways are candidates for reprogramming dynamics (Yildirim 2012). It has been proposed that some (if not all) of the reprogramming factors might be dispensable, when the somatic cells have high expressions of factors (Aoi et al. 2008; Huangfu et al. 2008; Li et al. 2009). Although we have not detected Sox2 expression in our parental cells, Liu et al. have shown Oct4, Sox2, and cMyc, and Kerkis et al. have shown Oct4 and Nanog expressions in DPSC (Kerkis et al. 2006; Liu et al. 2011).

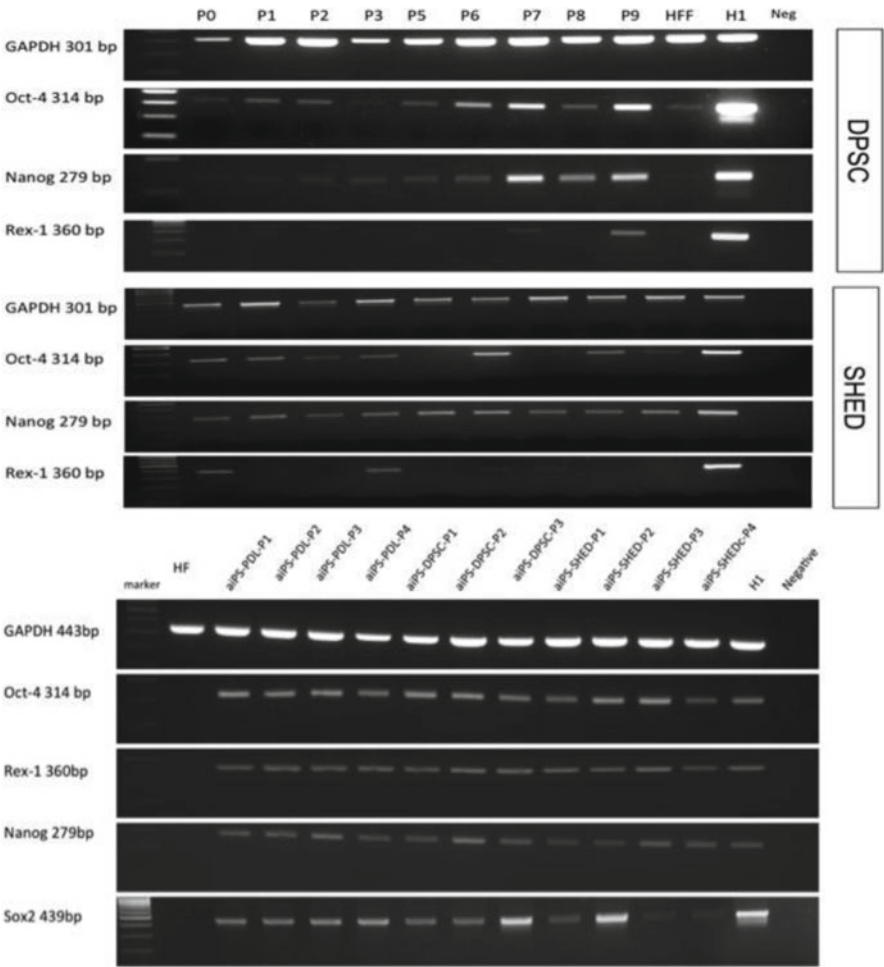


Fig. 7.2 Expressions of pluripotency markers by DPSC and SHED (on top), and derived iPS (on the bottom) (HFF human fetal fibroblasts, H1 human embryonic stem cell line)

Wnt, TGF β /Activin/Nodal and BMP signaling have important roles in induced reprogramming (Yildirim 2012). A downstream effector of the Wnt pathway, an inhibitor of glycogen synthase kinase-3 (GSK3), has been shown to maintain pluripotency in human and mouse ESCs (Sato et al. 2004). Moreover, Oct 4 interacts via the Wnt/ β -catenin signaling pathway and those events are involved in the maintenance of stem cell integrity and cell fate decision regulation (Abu-Remaileh et al. 2010). Canonical Wnt signals are transduced through Frizzled receptor and LRP5/LRP6 coreceptor to down-regulate GSK3, and my RT-PCR screening data on dental pulp tissue gene expression showed that permanent and deciduous dental pulp tissues express LRP5 and Jagged 1 (data not shown), which has been reposted as a β -catenin target gene required for ectopic hair follicle formation in adult epidermis (Estrach et al. 2006).

As one of the most studied pathways, TGF- β /Activin/Nodal plays important roles in the maintenance of the pluripotent state, and tooth development includes almost all members in this pathway (Thesleff et al. 1995). Dental pulp expresses abundantly TGF β family members (Gu et al. 1996; Sloan et al. 1999; Yildirim et al. 2008).

p53 acts as a barrier to somatic cell reprogramming (Kawamura et al. 2009; Marion et al. 2009; Utikal et al. 2009). It has been suggested that rather than silencing the p53 pathway, selecting target cells with lower levels of p53 and/or higher proliferative ability might give more successful reprogramming (Tapia and Scholer 2010). In dental pulp, p53 could not be detected immunohistochemically (Piattelli et al. 2000).

To minimize or eliminate genetic alterations in the derived iPSC line creation, factor-free human iPSC are necessary. At that rate, dental pulp cells with high proliferation ability and no p53 expression would contribute seamlessly to reprogramming events by the inhibition of TGF β and BMP signaling (James et al. 2005; Xu et al. 2008), and in addition, Wnt members, such as Wnt3a (Marson et al. 2008), in culture conditions.

7.1 Is Reprogramming Necessary for Dental Regenerative Therapies?

It has been questioned since the emergence of iPSC in cell biology, whether it is necessary to reprogram cells back to the pluripotent stem cell state for regenerative therapies or not. Pluripotency may well not be a prerequisite for the generation of certain differentiated cell types. This groundbreaking discovery of reverting the cellular clock back into the original position may help to dig deeper into cellular differentiation mechanisms. Moreover, modeling many human diseases might be possible by the discovery of cell-type-specific lineage reporters, lineage-tracking tools, and tools to disrupt, repair, or overexpress genes (Saha and Jaenisch 2009). If iPSC technology can provide a step-back system reversing the development, during which there is a gradual loss of differentiative potency proceeding from totipotency to pluripotency and multipotency in committed cell lineages toward terminal differentiation (Hemberger et al. 2009), we would meet with a ground state model that can serve as an observation tower for differentiation and lineage commitment. Since dental pulp has a very heterogeneous cell population including connective tissue cells, unique odontoblasts, immune cells, cementoblasts, and bone cells in a very rich neuronal and vascular environment, pushing ontogenically closed cells back to a common progenitor base (mesectodermal cells/NC cells?) would provide an opportunity for following the pathways through cellular specification. Another possible way to use dental pulp iPSC may be through mimicking epithelial–mesenchymal interactions. Dental pulp contains DPSC, which can differentiate to epithelial cells (Marchionni et al. 2009; Nam and Lee 2009; Sakai et al. 2010; Karaoz et al.

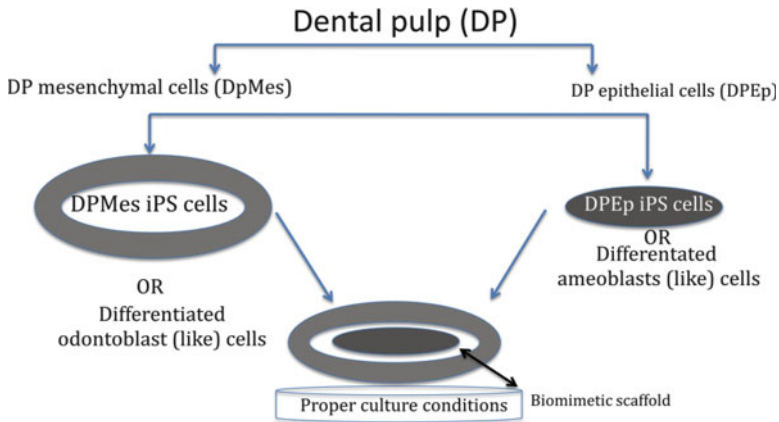


Fig. 7.3 A working hypothesis for epithelial-mesenchymal interactions to mimic dental development

2011; Zhao et al. 2012). After dissociation of epithelial and mesenchymal fractions, these cells can be reprogrammed into dental epithelial-iPSC and dental mesenchymal-iPSC. Since during the early passages of iPSC their epigenetic memories are retained (Kim et al. 2010a, b), these two pluripotent populations can be cocultured with a biomimetic scaffold between them in order to obtain developmental epithelial-mesenchymal interactive events (Fig 7.3).

Currently there are efforts being made to develop tissue-engineered tooth-like structures using mouse iPSC (Wen et al. 2012). Moreover, Arakaki et al. cultured mouse iPSC with mitomycin treated (inactivated) rat dental epithelial lines and obtained ameloblast-like cells (Arakaki et al. 2012).

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Chapter 8

Immunomodulatory Effects of DPSC

In addition to regenerative properties, the immunoregulatory properties of MSC have been demonstrated both in vivo and in vitro. Because of the low expression of MHC class II and co-stimulatory molecules, they are immunoprivileged cells. MSC can modulate the function of immune cells with different pathways of the immune response by means of direct cell-to-cell interactions and soluble factor secretions. They display immunosuppressive effects in a number of situations. In vitro, MSC inhibit cell proliferation of T cells, B-cells, natural killer cells, and dendritic cells. The immunomodulatory effect of MSC is mediated by a nonspecific anti-proliferative action of these cells, and it is dependent on cell-to-cell contact or secreted soluble factors such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E2, nitric oxide (NO), HLA-G, transforming growth factor (TGF)- β , interferon (IFN)- γ , and interleukin (IL)-1 β (Nasef et al. 2008; Shi et al. 2011; De Miguel et al. 2012).

For the first time Pierdomenico et al. demonstrated that DPSC display an increased immunosuppressive activity when compared to bone marrow MSC (Pierdomenico et al. 2005). Although the regulatory mechanism has not been surrogated, Huang et al. showed that the implantation of DPSC derived from rhesus monkeys into the hippocampus of mice did not cause any immune rejection (Huang et al. 2008a, b). Later on Tomic et al. showed DPSC suppressed proliferation of peripheral blood mononuclear cells and that treatment with toll-like receptor 3 and 4 agonists augmented this suppressive potential (Tomic et al. 2011).

The HLA-G nonclassical MHC class I molecule was originally described in first-trimester trophoblasts at the fetal–maternal interface, and HLA-G5 is one of the major isoforms described in healthy tissues comprising trophoblast, thymus, cornea, erythroid, and endothelial precursors (Menier et al. 2010; Fainardi et al. 2011). I observed that dental pulp expresses HLA-G5 (data not shown). Deciduous dental pulp may well have an intrinsic mechanism in order to manage resorption events. This relates to the hypothesis mentioned earlier that SHED might have an immunoregulatory function to modulate resorption events. It would be valuable to see if as per the proposal of Rizzo et al. that the evaluation of sHLA-G production in IL-10-treated bone marrow-MSC cultures is a possible marker of immunoregulatory function (Rizzo et al. 2011), would valid for SHED as well.

It is clear that there are still important issues regarding the therapeutic usage of stem cells. It has been reported that any transplanted stem cell-derived tissues, if not genetically identical to the recipient, has the potential to induce allograft rejection via “indirect” recognition, since the absence of the expression of HLA molecules cannot prevent immunological rejection because of activation of NK cells (Bryceson and Long 2008; Taylor et al. 2011).

Although it is still unclear whether any of the MSC can be used for future cell therapies, establishing stem cell banks containing pluripotent cells which closely match or are compatible to HLA along with the induction of antigen-specific immunological tolerance instead of requiring lifelong immunosuppressive therapies are hopes for the avoidance of immunological rejection (Taylor et al. 2011). There are efforts to establish clinical-grade DPSC banks with a sufficient repertoire of HLA types. Tamaoki et al. used DPSC to generate iPSC banking with a sufficient repertoire of HLA types. The practical isolation and handling of dental pulp cells may make it easy to expand the size of the bank in multiple institutes (Tamaoki et al. 2010).

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Chapter 9

Dental Pulp Is a Complex Adaptive System

The damaged or lost structure of teeth caused by caries or trauma can be treated clinically by replacement with an artificial material (fillings) and the outcome is usually satisfactory. Yet, tooth loss is the most common organ failure. The complex biological structure of the tooth organ including the enamel, dentin, cementum, and pulp create obstacles for any ambitious dream of the substitution of artificial material with a bio-tooth. Although the focus of the most regenerative studies is toward achieving a means to repeat evolutionary signals secondarily and initiate development secondarily, lack of understanding of the nature and interactions of tooth creating cells blocks this goal. Efforts to discover possible lineage-specific propensities within one group of cells are progressing more favourably. Although stem cells have been taken out of the body and manipulated ex vivo, there are many steps for successful cell-based therapies due to committed cells being unable to orchestrate the regeneration of complex tooth structures (Yildirim et al. 2011a).

Regeneration of dental pulp, dentin, periodontium including cementum, periodontal ligament, and alveolar bone, and regeneration or synthesis of enamel-like structures have been reported (Zeichner-David 2006; Foster et al. 2007; Huang et al. 2008a, b; Huang 2009; Kim et al. 2010a). Moreover, regeneration or de novo formation of an entire, anatomically correct tooth has been shown (Ikeda et al. 2009; Kim et al. 2010a, b). However, there is no clinical outcome yet, since the difficulties in obtaining proper cells (embryonic tooth bud cells, allogenic human cells, xenogenic embryonic tooth bud cells), and cell delivery approaches (scaffolds), cause the signaling molecules to encounter several translational barriers. In summary, albeit considerable progression for teeth regeneration with both cell transplantation and cell homing approaches has been achieved, we still need more tangible methods and approaches toward clinical translation (Yildirim et al. 2011a).

9.1 Changing Concepts in Cell Biology

The central dogma of molecular biology states that the molecular basis of genetic inheritance is established by a linear relationship between genetic information encoded in DNA and translated protein (DNA → mRNA → protein) (Li and Xie 2011; Huang

2012). However, with the aid of advanced single-cell fluorescence microscopy, transcriptomes and proteomes have been quantified with single-molecule sensitivity showing that the mRNA copy number does not correlate with the protein copy number for the same gene in an *E. coli* cell (Yu et al. 2006a, b; Taniguchi et al. 2010). There is accumulating evidence showing that genotype might not be translated in an obvious manner into a corresponding phenotype and genes are not even the sole basis of inheritance. In conclusion, “the complexity in the relationship between genotype and phenotype has emerged with inescapable clarity” (Huang 2012).

9.2 The Tooth as a Complex Adaptive System

Since many phenomena in life are neither linear nor able to be reduced to their simplistic units (or both), the inability of linear analysis to explain nonlinear systems has been accepted at the beginning of 1900s. A nonlinear system is more than the sum of its parts and small inputs can have large system effects (or vice versa) and there is sensitivity to initial conditions, which makes prediction almost impossible (Waldrop 1992).

Aydinoglu stated that:

The problem in working with non-linear systems was beyond human computational ability. When non-linear relations are realized, the related data was not preserved and/or the non-linear relations cannot be measured/calculated due to their complexity. This happens because “modeling the nonlinear outcomes of many interacting components has been so difficult that both social and natural scientists have tended to select more analytically tractable problems” (Anderson 1999), which produces deficient and incomplete reflections of reality. Thus, scholars end up having a discipline that is not connected to life and it does not help us to control or predict phenomenon as a result of its dependency on linear modeling.

When a part of a system is examined by itself, it means it is isolated from the other parts in the system. Hence, the analysis will be flawed because the interactions between the parts are going to be missed. Moreover, feedback loops could weaken or strengthen certain behaviors, and processes are going to be missed. These loops are important because they directly affect the equilibrium of the organization (Aydinoglu 2010).

These statements can be effectively applied to “tooth organization” since teeth behave like any complex adaptive systems (CAS).

A complex adaptive system consists of a large number of agents, each of which behaves according to some set of rules. These rules require the agents to adjust their behavior to that of other agents. In other words, agents interact with, and adapt to, each other (Stacey 2003).

If teeth behave like a CAS, it should demonstrate basic features of such systems. According to Aydinoglu, the characteristics and principles of CAS are components/agents, variation and diversity, interaction and interdependency, feedback, unpredictability and nonlinearity, the edge of chaos, self-organization and emergence, adaptation and learning, historicity and path-dependence, and finally coevolution (Aydinoglu 2011). Although regenerative, molecular, microbiological, tissue organization, and several other aspects of a tooth system should be analyzed individually, I will attempt to demonstrate how teeth conform to the basic features of CAS.

1. *Large numbers of components*: Teeth have various cells and tissues.
2. *Variation and diversity*: Each cell different from each other and its performance depends on the other cells.
3. *Interaction/connectivity/interdependence*: Homeostasis established via interaction/connectivity of the different elements of a tooth system. However, every single part of the tissue (enamel, dentin, etc.) has interdependent cells (ameloblasts, odontoblasts, etc.).
4. *Feedback*: Positive (amplifying effect) feedback to periodontal attachment occurs during eating an apple and negative (dampening affect) feedback is sent via infected dentinal tubules to the dental pulp.
5. *Unpredictability and nonlinearity*: If you do not brush your teeth regularly, caries MIGHT develop. Oral habits MIGHT not cause pain at the beginning.
6. *The edge of chaos/far from equilibrium*: Being away from equilibrium (through poor oral health or continuous trauma) might alter the environment to create a better configuration that increases the likelihood of its survival (emergence resistant biofilms).
7. *Emergence, self-organization-strange attractors*: Reparative dentin emergence cannot be reduced to a single molecule. Many constituents have many diverse functions along the process.
8. *Adaptation to environment*: Teeth are quite resilient to threats and adaptive to their environment. There is a dynamic between health and disease.

As a conclusion, with the help of emergent concepts of complexity, we can explore the properties and characteristics of tooth functions from a complex adaptive systems perspective. Attaining to the desired dentin-specific marker(s) for characterization of the nature of the replacement cell population responsible for forming reparative dentin seems handicapped since the linear gene protein arrow is preparing to give its place to more complex biological systems.

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Chapter 10

Conclusions

The mesectodermal origin of DPSC makes them a strong source of raw material for various human tissue regenerations in the future. Yamanaka's group generated iPSCs from DPSC of two putative HLA-homozygous donors who match ~20 % of the Japanese population at major HLA loci (Okita et al. 2011). The recognition of DPSC–iPSCs infers their availability to begin exploring their therapeutic potential at the pre-clinical level. However, using them in the area of tooth regeneration and making bio-teeth with all dental, and oro-musculatory and supportive tissue functions seems to be much more complicated. Currently, DPSC-based therapies are offering functional, vascularized, and innervated non-mineralized pulp tissue constructions (Huang et al. 2010; Kim et al. 2010a, b; Iohara et al. 2011; Yang et al. 2012). Furthermore, the stimulation of stem cell niches for odontoblast regeneration warrants further studies. With the help of current emergent concepts of complexity, we can explore the various functions of tooth tissues from a complex adaptive systems perspective.

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About the Author

Sibel Yildirim is professor at the Faculty of Dentistry, Department of Pediatric Dentistry, University of Selcuk, Konya, Turkey. She has been a lecturer and a practising dentist at the university for years. She and her colleagues have established the first multidisciplinary, dental/oral research centre in Turkey. She has authored many papers in international journals and has completed several research projects. Along with her pediatric dentistry Ph.D., she has also her second Ph.D. on histology and embryology. Her research interests include many aspects of dental pulp and induced pluripotent stem cells, deciduous tooth resorption, the viral ethiopathogenesis of pulpal/periapical diseases of deciduous teeth, and vital regenerative pulp therapies using recombinant human proteins or gene therapy. Accordingly, she has led some experiments on deciduous tooth pulp tissue and her team obtained important results showing high regulatory capacity of dental pulp cells on the events of deciduous tooth resorption or retention.

She had a chance to study with the indisputable leaders in the dental engineering field in Japan, Switzerland, and the USA. In addition to her dental tissue engineering studies, she has pursued reprogramming experiments. She could observe that stem cells that have dental origin displayed strong potential for reprogramming. She believes comprehensively that isolating epithelial and mesenchymal stem cells from deciduous teeth and turning them to iPSCs will point toward novel venues for in situ restoration of dental tissue repair. Currently her ongoing projects are focused on epigenetic regulations in dental pulp tissue.

As a pediatric dentist, histologist, and embryologist, after long years of experience in academic fields in different continents, she has found herself that she became a student again who is keen to reach beyond the traditional boundaries of biology. After she met up with Sui Huang's and Stuart Kaufman's marvellous ideas, she totally convinced that complexity is the only field that helps to achieve her goals, moving one step closer to understanding life.

Index

C

Cell culture media, 42, 44–48
Complex adaptive systems, 75–77, 79
Cryopreservation, 49

D

Deciduous dental pulp stem cells, 22, 30, 32, 43, 64, 73
Deciduous teeth, 13, 21, 22, 28–33, 36, 41, 43, 48, 56
Dental evolution, 1–3
Dental pulp,
Dental pulp stem cells (DPSC),
Dentin, 1, 5, 8, 9, 12, 13, 17–22, 25–27, 29, 31, 32, 35, 36, 48, 56–58, 60, 75, 77
Differentiation, 6, 8–13, 18, 19, 26–29, 35, 36, 41, 43–45, 47, 48, 54–60, 68
DPSC. *See* Dental pulp stem cells (DPSC)

E

Enzyme digestion, 42, 43
Epigenetic modifications, 36

I

Immunomodulation, 73–74
Induced pluripotent stem cells (iPSC), 65–69, 74, 79
iPSC. *See* Induced pluripotent stem cells (iPSC)
Isolation methods of dental pulp stem cells, 41–49, 54

M

Multidifferentiation, 54–60

N

Niche, 11, 12, 27, 28, 33–36, 79

O

Odontoblast differentiation, 9–12, 19, 35, 36, 56, 58–60
Odontoblast-like cells, 25–26, 45, 47, 57–59
Outgrowth method, 42–45

P

Permanent tooth, 21, 22, 28, 36, 41, 56
Phenotypic characteristics, 53–54
Pluripotent cells, 63, 74

R

Reprogramming, 65–69
Resorption of deciduous teeth, 21, 22

S

SHED. *See* Stem cells from human exfoliated deciduous teeth (SHED)
Stem cells,
Stem cells from human exfoliated deciduous teeth (SHED), 28, 29, 31, 32, 43, 45, 47, 48, 53, 54, 56, 57, 65–67, 73

T

Teeth development, 5–14, 35, 59, 68

U

Undifferentiated mesenchymal cells, 9, 20, 26